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Synthesis of 11-Hydroxyl *O*-Methylsterigmatocystin and the Role of a Cytochrome P-450 in the Final Step of Aflatoxin Biosynthesis

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Abstract: The major skeletal rearrangements (anthraquinone → xanthone → coumarin) that occur in the complex biosynthesis of aflatoxin B_1 are mediated by cytochromes P-450. Previous experiments have suggested that two successive monooxygenase reactions are required to convert the xanthone *O*-methylsterigmatocystin (OMST) to aflatoxin, a process we demonstrate is mediated by a single P-450, OrdA, in *Aspergillus parasiticus* in accord with findings in *A. flavus*. The first oxidative cycle is proposed to result in the formation of 11-hydroxy *O*-methylsterigmatocystin (HOMST), while the second entails aryl ring cleavage, demethylation, dehydration, decarboxylation, and rearrangement to give aflatoxin − a remarkable sequence of transformations. To test this hypothesis, HOMST has been synthesized by an alkylnitrilium variant of the Houben−Hoesch reaction. The troublesome xanthone carbonyl was protected as a butylene to allow further elaboration of the molecule, and then the product xanthone was restored in a uniquely facile peracid deprotection. Methods were devised to construct the sensitive dihydrobisfuran and to maintain the oxidation state of the partially methylated hydroquinone. Expression of *ordA* in a yeast membrane preparation enabled the intermediacy of HOMST both to be detected in the conversion of OMST to aflatoxin and to be established directly in the biosynthesis of the mycotoxin. Having secured the role of HOMST in aflatoxin formation, the mechanism of the second oxidative cycle of this P-450 is considered.

Introduction

Aflatoxin B_1 (**4**, AFB_1) is a widespread contaminant of foodstuffs, especially in equatorial parts of the world where it has been correlated to an increased incidence of liver and kidney tumors.[1−3] This environmental carcinogen is produced by strains of the fungal genus *Aspergillus* and is notable for its complex biosynthesis.[4] In particular the post-polyketide synthase steps are marked by skeletal rearrangements in which the first-formed anthraquinone, norsolorinic acid (**1**), is transformed to the dihydrobisfuran-containing versicolorin A (**2**), which then is oxidatively rearranged to the xanthone demethylsterigmatocystin (**3**, R = R′ = H).[5] After stepwise O-methylations to *O*-methylsterigmatocystin (**3**, OMST; R = R′ = Me),[4,6,7] recent genetic evidence suggests that a *single* cytochrome P-450 converts this penultimate intermediate to AFB_1, a process involving net oxidative cleavage of the xanthone A-ring, O-demethylation, dehydration, decarboxylation, and rearrangement (vide infra and Scheme 1).[8]

Insight into the latter coumarin-forming process can be gathered from a diverse set of observations. First, deuterium from isotopically labeled sodium acetate was found in expected positions in the A-ring of sterigmatocystin (**3**, ST; R = Me, R′ = H),[9] but on adjacent carbons in the cyclopentenone ring of AFB_1 (**4**, Scheme 1).[10] It can be deduced from these separate experiments that epoxidation of the A-ring of **3** could lead to a NIH shift and formation of the previously unknown 11-hydroxy OMST (**6**, HOMST; Scheme 2). Second, consistent with oxidative aromatic cleavage at this site in the A-ring of **3**, $^{18}O_2$ (O*) was found to be incorporated at C-1 and elsewhere in AFB_1.[11] Third, the identity and oxidation state of the carbon lost in the overall process of coumarin formation were shown to be specifically C-11 (•, Scheme 1), which was trapped in the correct stoichiometry exclusively as carbon dioxide.[12] For this oxidation state to be achieved, two cycles of monooxygenase chemistry must be involved. Finally, while the number of

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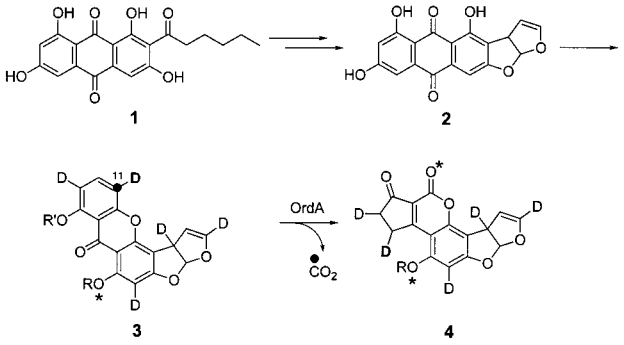
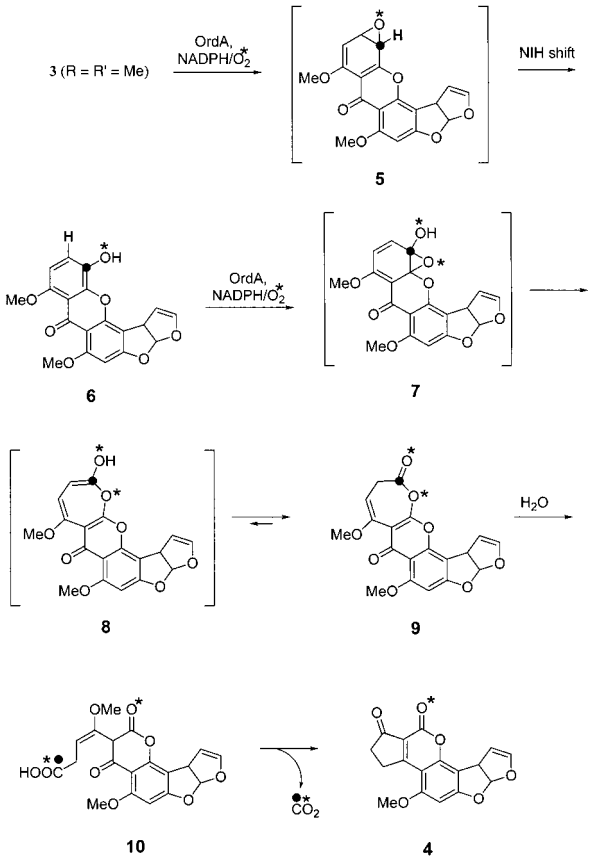
Scheme 1



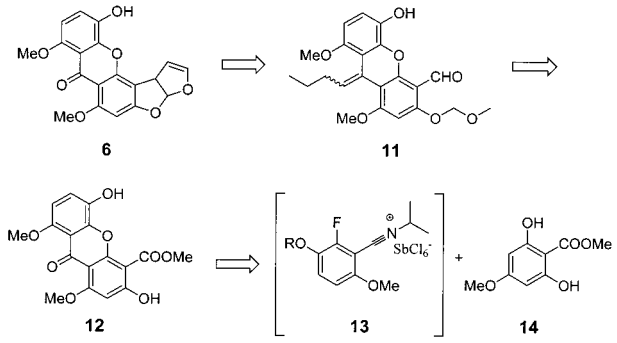
Scheme 2



enzymes required for the conversion of OMST (**3**, R = R′ = Me) to AFB_1 (**4**) was initially thought to be larger, the remarkable finding of Prieto and Woloshuk[8] in the related organism *A. flavus* clearly pointed to a single cytochrome P-450 carrying out this multistep oxidative cleavage and molecular reorganization. To accommodate all of these observations, we have proposed the following mechanism to account for this remarkable sequence of events as shown in Scheme 2.[4,11]

The first cycle of P-450 mediated oxidation is proposed to give aryl epoxide **5**, which would give the previously suggested NIH shift to 11-hydroxy OMST (**6**).[10] The second cycle of oxidation can be visualized to occur in several ways. One of these (Scheme 2) involves further epoxidation to **7** and rearrangement to the oxepin **8**, which would rapidly tautomerize to the hydrolytically unstable 7-membered lactone **9**. Opening of the lactone, demethylation, reclosure, and decarboxylation in some unspecified order can be invoked to give AFB_1 (**4**). The intermediacy of HOMST (**6**) is central to this proposed

Scheme 3



mechanistic scheme. In this paper, we describe the total synthesis of this putative intermediate by extension of our previously described N-alkyl nitrilium route to highly substituted xanthones.[13] To provide a biochemical system to examine the multifold reaction of this P-450 in isolation from the other proteins of the aflatoxin biosynthetic pathway, we have cloned the *ord1* analogue from *A. parasiticus*, *ordA*, and achieved its functional expression in yeast.

Results

Synthesis of 11-Hydroxy *O*-Methylsterigmatocystin. Synthesis of the fused dihydrobisfuran-containing intermediates of AFB_1 biosynthesis requires either early installation of the bicycle in an aryl precusor prior to xanthone formation or its construction subsequently. The lability of the bisfuran (a masked dialdehyde) to acidic and basic reaction conditions thwart the former approach. Alternatively, assembly of the dihydrobisfuran after xanthone formation must contend with the substantial electrophilicity of the xanthone carbonyl.[13] We have devised a general solution to the preparation of highly substituted xanthones which first takes advantage of the reactivity of N-alkyl nitrilium salts as **13** (Scheme 3) in a Friedel−Crafts sense to give xanthones as **12**.[13] Second, protection of the xanthone carbonyl as its corresponding butylene **11** can be simply achieved by reaction with *n*-butyllithium. A wide variety of transformations can then be carried out to elaborate the desired substitution followed by a uniquely facile deprotection to yield the xanthone product. The retrosynthesis of 11-hydroxy *O*-methylsterigmatocystin (**6**, HOMST) is outlined in Scheme 3.

Considerable experimentation led to the choice of benzonitrile **18** (Scheme 4) as the nitrilium precursor, which was prepared in five steps from commercially available acetophenone **15** in 44% overall yield. Baeyer−Villiger oxidation, hydrolysis of the resulting acetate, and SEM protection afforded **16** (87% yield) by adaptation of a published method.[14] Directed metalation of **16** at low temperature occurred specifically at C-3 and allowed the introduction of nitrilium precursors from, for example, an alkyl isocyanate, or, finally, directly from phenylisocyanate. Attempted generation of the nitrilium salt **13** (Scheme 3) from imidoyl chloride **19**[15] and reaction with **14** proceeded poorly. Similarly, von Braun dehydration[16] of **20** failed presumably

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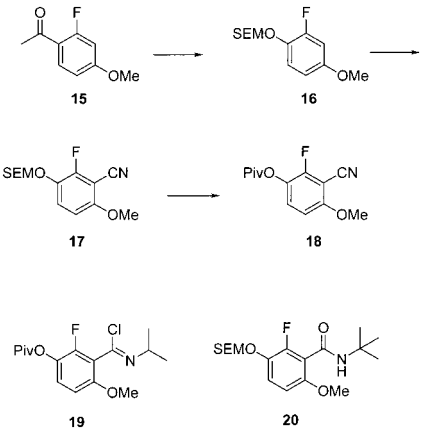
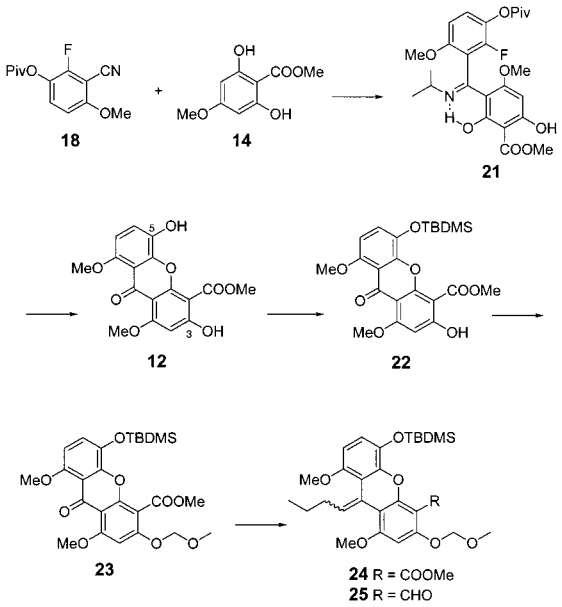
Scheme 4



Scheme 5

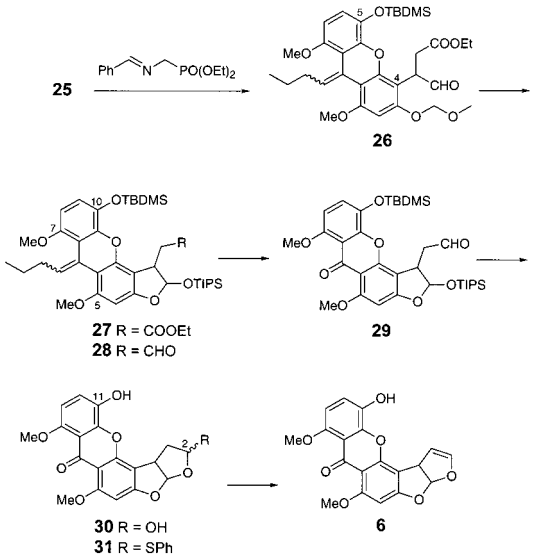


owing to decomposition of the SEM group. Metallation of **16** followed by treatment with phenylcyanate[17] and introduction of the pivaloyl group, however, gave benzonitrile **18** in >75% yield. These reactions were readily scalable to allow 10 g quantities of this key starting material to be prepared.

Pivaloyl protection rendered the phenol group more electron-withdrawing and, therefore, enhanced the electrophilicity of the nitrilium salt **13**, which was most efficiently prepared by $SbCl_5$ reaction with the aryl nitrile **18** and 2-chloropropane. When the nitrilium salt of **18** (**13**) and **14** (Scheme 5) were reacted in a 1:1 ratio, isopropylimine **21** was obtained in about 50% yield accompanied by approximately 10% of this product lacking the pivaloyl group. Reasoning that loss of this protecting group would slow the rate of electrophilic aromatic substitution by the nitrilium ion and itself lead to side reactions, the ratio of the reaction partner **14** was increased (**14**:**18**, 2.5:1), and the time of reaction was halved to now afford **21** in 90% yield and <5% of the depivaloyated material. Alkaline hydrolysis readily afforded the xanthone **12** (Scheme 5).[18,19]

The sparingly soluble **12** could not be selectively O-protected with MOMCl at C-3 or by TBDMSCl at either C-3, or C-5.

Scheme 6



However, both phenols could be readily silylated, and, owing to participation by the adjacent carbomethoxy group, the TBDMS at the C-3 hydroxyl could be rapidly and specifically removed by treatment with HF (95% yield). The C-3 hydroxyl was methoxymethylated to **23** in essentially quantitative yield. Prior to construction of the dihydrobisfuran, the xanthone carbonyl was masked as an isomeric mixture of butylenes **24** by the low-temperature addition of butyllithium and dehydration induced during silica gel chromatography.[13]

With the substituted xanthone completely protected, the methyl ester **24** was converted to the corresponding aldehyde **25** by low-temperature DIBAL reduction followed by TPAP/NMO oxidation[20] in excellent yield over the two steps. The Martin geminal disubstitution protocol[21,22] is well-suited for assembly of the required carbon skeleton and proceeded in 75−80% yield to **26**. Treatment of **26** with TIPS triflate according to the method of Whittamore et al.[22,23] smoothly afforded the mixed acetal **27**, which was directly reduced to aldehyde **28** with DIBAL (90% yield).

In the presence of the unprotected aldehyde, **28** was treated with *m*-CPBA to selectively restore the xanthone carbonyl in **29** as the only detectable product, attesting to the usefulness of the butylene protection/deprotection tactic. As anticipated,[22] fluoride-mediated desilylation afforded the hemiacetals **30**, which were converted to the thiophenyl acetals **31**.[24] Oxidation with *m*-CPBA at −45 °C gave a diastereotopic mixture of sulfoxides, which was pyrolyzed in a preheated bath (110−120 °C) to generate (±)-HOMST (**6**). The total synthesis of **6** required 14 linear steps from arylnitrile **18** and was completed in >13% overall yield (Scheme 6).

Expression of *ordA* in Yeast. The *A. parasiticus ordA* gene, identified previously by Bhatnagar,[25] and independently in our group (Minto, R. E.; Townsend, C. A., unpublished), was

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amplified by PCR from wild-type *A. parasiticus* cDNA, and ligated into the multiple cloning site of the commercially available *S. cerevisiae* expression vector pYES2 (Invitrogen), yielding the final *ordA* expression vector pYCapoKX. Transformation into a suitable host, Invitrogen INVSc2 *S. cerevisiae* cells, was confirmed by reextracting plasmid DNA from yeast transformants, and retransforming and screening *E. coli*. The yeast transformants were best maintained on minimal medium plates supplemented with glucose, which represses expression of *ordA* under the control of the pYES2 GAL1 promoter,[26,27] but conditionally selects for uracil utilization by complementation with the pYES2 *ura3* selection marker.[28]

Expression of *ordA* was achieved by growth of transformants in YNB medium containing 2% galactose as the sole carbon source to induce the GAL1 promoter. We attempted to quantify the amount of OrdA produced by two methods. Induced transformants were disrupted with glass beads, and after analysis of the soluble extract by SDS−PAGE we could see no induction of the 60 kD OrdA. Eukaryotic P-450s are usually tightly membrane bound[29] owing to an N-terminal membrane anchor sequence (also present in OrdA), and so we assumed that the protein remained bound to the membrane in its yeast host. Second, we attempted a carbon monoxide binding assay. This is a well-known technique for detection of P-450s,[30] whereby CO gas is bubbled through a protein extract or live cell culture, binding irreversibly to the heme iron and producing a characteristic absorption at 450 nm, which correlates with P-450 concentration. However, P-450 expression could not be detected above background levels of absorption in our host by this technique.

The sole means of detection of OrdA was by catalytic activity. Incubation of small amounts of OMST with live yeast transformants grown in galactose produced aflatoxin B1, the blue fluorescent signature of which was unambiguously detectable by TLC. The same host cells transformed with empty pYES2 vector did not produce aflatoxins, nor did either of these transformants when grown in a glucose-containing medium, repressing expression under control of the GAL1 promoter.

To facilitate purification of OrdA we attempted to overproduce an N-terminally truncated and soluble protein, an approach that has been used successfully in the past.[31,32] The location and extent of the N-terminal membrane anchor in OrdA was identified by sequence alignment with other P-450s for which this information is known,[33−36] and by comparing secondary structure predictions[37,38] of eukaryotic P-450 N-termini. A shortened form of the gene, which resulted from deletion of amino acids 3−20, was amplified by PCR and ligated into pYES2. When induced under identical conditions to those described above for wild-type OrdA, we found that the modified protein continued to catalyze the conversion of OMST to AFB_1, but the enzyme activity was exclusively membrane associated, presumably owing to other membrane-binding residues, which has since been documented.[31]

OrdA-Mediated Conversion of OMST and 11-Hydroxy OMST to AFB_1. Small-scale expression was used to screen pYCapoKX-transformed yeast colonies, or otherwise verify OrdA activity before large-scale expressions. Larger scale cell-free extracts were used for the purification or analysis of potential intermediates in the OMST to AFB_1 conversion. We found that by using this method we were able to retrieve more of the final product and untransformed starting compound than by simply administering the synthetic compounds to cultures of whole cells.

As expected, OrdA appeared firmly membrane-bound. When induced yeast cells transformed with pYCapoKX were disrupted, activity could only be found in the membrane fraction. To rule out the necessity of insoluble coenzymes, inactive yeast membranes from pYES2 transformants were mixed with the soluble fraction from a cell-free preparation. If OrdA were soluble or partially soluble, it could function with the hypothetical membrane-bound yeast coenzyme. Only the induced and transformed yeast cell membranes exhibited the ability to convert OMST to AFB_1, and activity was relatively high as compared to that of whole cells, although only for 24−48 h. The addition of NADPH to the cell-free system did not observably improve enzyme activity. This implies that all necessary coenzymes (specifically cytochrome P450 reductase[39]) and cofactors (NADPH, FAD, and FMN) are sufficiently bound to the yeast cell membrane or to other membrane-bound enzymes after lysis to sustain the observed chemistry.

By using this cell-free extract, we were able to duplicate in *A. parasiticus* the observed production of AFB_1 from OMST in *A. flavus*,[8] and we have extended it to monitor AFB_1 production from synthetic HOMST. Moreover, with the added sensitivity of the cell-free system, we have been able to monitor transient production of HOMST from OMST. These results are shown in Figure 1. To control for degradative side products that might occur during the incubation, we placed synthetic standards of OMST, HOMST, and AFB_1 in buffer under conditions identical to the yeast incubations (lanes 1, 2, and 5). We observed little decomposition of OMST and AFB_1, while HOMST was less stable. After incubation of OMST with *ordA*-expressing yeast (lane 3), the unmistakable bright blue fluorescence corresponding to AFB_1 appeared at the appropriate R_f. Moreover, we observed the formation of a second compound corresponding to HOMST, strongly supporting its intermediacy in this conversion. When HOMST was incubated with *ordA*-expressing yeast (lane 4), again production of AFB_1 was seen. Conversely, when OMST and HOMST were incubated with pYES2 (vector alone) transformants (lanes 6 and 7), no AFB_1 production was observed.[40]

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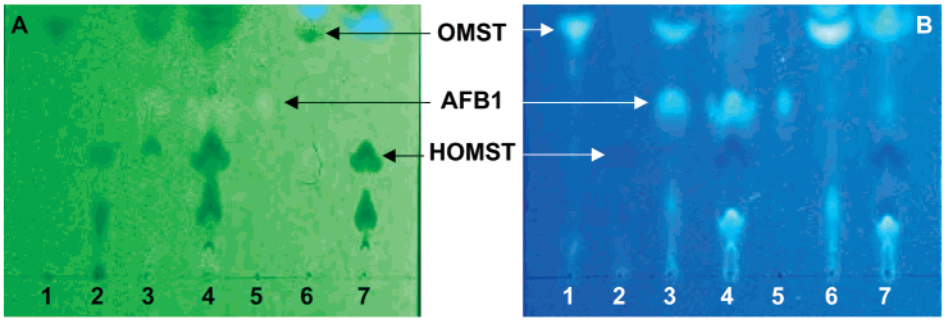


Figure 1. Thin-layer chromatographic analysis of the conversion of OMST and HOMST into AFB_1 under (A) long wavelength UV light, and (B) short wavelength UV light. Lane: (1) OMST standard; (2) HOMST standard; (3) OMST incubated with pYCapoKX-transformant cell free extract; (4) HOMST incubated with pYCapoKX-transformant cell free extract; (5) AFB_1 standard; (6) OMST incubated with pYES2-transformant cell free extract; (7) HOMST incubated with pYES2-transformant cell free extract.

These findings were confirmed by HPLC, ELISA, or MALDI-MS experiments. Small amounts of AFB_1 produced from OMST or HOMST were detectable and separable from yeast metabolites by reverse phase HPLC, and the characteristic AFB_1 UV signature was observed. In addition, the putative AFB_1-containing fractions were collected, lyophilized, and confirmed by TLC. While readily detectable by fluorescence quenching on TLC, HOMST could not be observed as an intermediate in the OMST to AFB_1 reaction by HPLC, presumably because the transient amounts produced were simply below the limits of UV detection. It was detectable by MALDI-MS, however, after partial purification by preparative TLC to give an M + H ion at *m*/*z* 355.24, while an authentic sample of HOMST gave the corresponding ion at *m*/*z* 355.23. The results were further confirmed with a commercially available competitive-direct enzyme linked immunosorbent assay (CD-ELISA). We found the test kit to work well at aflatoxin levels as low as 10 ppb, and it uses a simple color test that did not significantly cross-react with OMST, HOMST, or other aflatoxin biosynthetic precursors available to us. Fluorescence, however, remains the best technique for sensitive detection of AFB_1, with an analytical limit on TLC in our hands as low as 0.3 ng.

Substrate Specificity of OrdA. Bhatnagar, Cleveland, and Kingston have previously shown that a partially purified enzyme complex was able to convert both OMST (**3**, R = R′ = Me, Scheme 1) and dihydro OMST (**32**, DHOMST) to AFB_1 (**4**) and AFB_2 (**33**), respectively, and that OMST was the preferred substrate for the reaction.[41] With our washed membrane system we had the opportunity to evaluate whether the single expressed P-450 was able to accept both dihydro- and tetrahydrobisfuran substrates, or if another gene product of the biosynthetic cluster was necessary for the reactions to proceed.

Dihydro-*O*-methylsterigmatocystin (**32**, DHOMST) and dihydro-11-hydroxy-*O*-methylsterigmatocystin (**34**, DHHOMST) were prepared from synthetic OMST and HOMST, respectively, by hydrogenation over Pd/C.[42] After incubation with cultures of pYCapoKX transformants grown in galactose, we found that AFB_2 was produced in a yield suitable for analysis by TLC and ELISA (Figure 2, lanes 8 and 10). While the amount of DHHOMST (**34**) available was limited, conversion to AFB_2 by the active cells was clearly detectable relative to the inactive control (lanes 7 and 8) and particularly in the CD-ELISA test (cf. lanes 7, 8, and b). As expected, sterigmatocystin (**3**, R = Me, R′ = H) was not observably consumed and produced no aflatoxins (lanes 5 and 6). Furthermore, after identical incubation, AFB_1 was not reduced to AFB_2 by either the P-450 or the

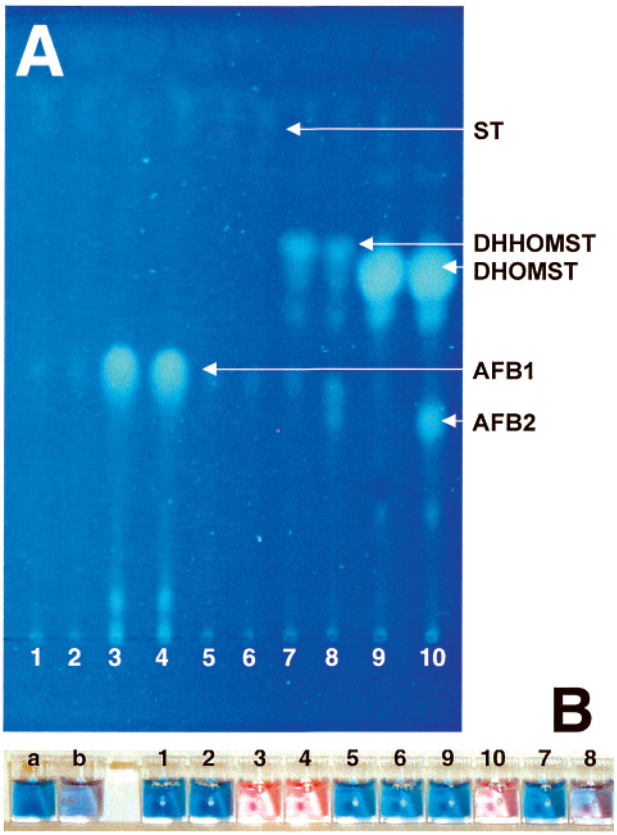


Figure 2. Conversion of DHOMST **32** and DHHOMST **34** to AFB_2 **33**. (A) TLC analysis of chloroform extracts of OrdA-active and -inactive yeast cell extracts. Lane: (1) Inactive yeast, no metabolites added;[40] (2) active yeast, no metabolites added;[40] (3) inactive yeast incubated with AFB_1; (4) active yeast incubated with AFB_1; (5) inactive yeast incubated with ST; (6) active yeast incubated with ST; (7) inactive yeast incubated with DHHOMST; (8) active yeast incubated with DHHOMST (AFB_2 has a slightly lower R_f than does AFB;[43] (9) inactive yeast incubated with DHOMST; (10) active yeast incubated with DHOMST. (B) Agri-Tox CD-ELISA screening kit results. Blue color indicates no aflatoxins; pink indicates the presence of aflatoxins. Lanes correspond to those in (A), and where control lanes are: (a) no aflatoxin; (b) 100 microliter 20 ppb AFB_1.

(40) It should be noted that the pale spots seen in the photograph at an R_f similar to AFB_1 were clearly visible to the eye as pale yellow metabolites that appeared in every yeast/chloroform extract, but were only visible when not masked by the bright fluorescence of AFB_1.
(41) Bhatnagar, D.; Clevelend, T. E.; Kingston, D. G. I. *Biochemistry* **1991**, *30*, 4343−4350.
(42) Chen, P. N.; Kingston, D. G. I.; Vercellotti, J. R. *J. Org. Chem.* **1977**, *42*, 359−3605.

yeast host cells (lanes 3 and 4). These results confirm previous findings that the latter stages of the aflatoxin biosynthetic pathway, specifically the steps beyond the "branch point" at which the tetrahydrobisfuran versicolorin B is oxidized to versicolorin A (**2**),[41,43,44] are controlled by the same enzymes, which only discriminate between the dihydro- and tetrahydrobisfuran-containing late intermediates to the extent of small kinetic differences in reaction rate.

Discussion

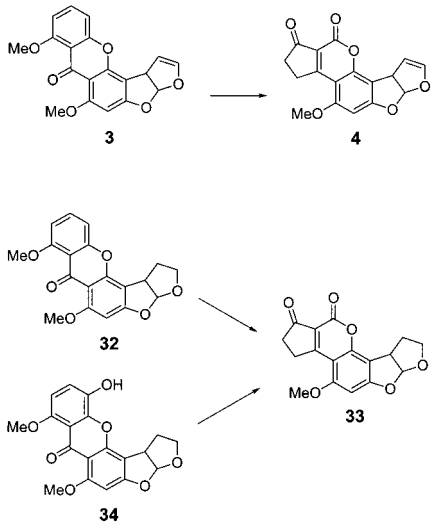
The formation of aflatoxin takes place through an unusually long and complex sequence of biochemical events. In particular, the nuclear reorganization of the initially formed anthraquinone (see Scheme 1) to xanthone intermediates and, finally, the coumarin of aflatoxin itself are all oxidative. Cytochrome P-450 enzymes mediate these rearrangements, which are exemplified in the biosynthesis of other fungal acetogenins suggesting a wider synthetic role for proteins of this superfamily.

As discussed at the outset, evidence for an apparent NIH shift and the stoichiometric loss of C-11 from OMST (**3**, R = R′ = Me; Scheme 2) as carbon dioxide point to two sequential monooxygenase steps in the formation of aflatoxin. We have demonstrated that this extensive structural change is catalyzed by OrdA, a single P-450 isolated from the *A. parasiticus* aflatoxin biosynthetic gene cluster in keeping with experiments in *A. flavus*.[8] We have proposed the intermediacy of HOMST (**6**, Scheme 2) as the product of the first monooxygenase reaction.[4] It is likely formed through the proposed epoxide **5** to account for the evident NIH shift.[10]

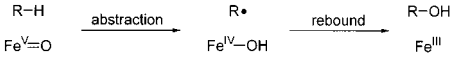
To establish the role of this proposed intermediate in the final steps to AFB_1, HOMST was prepared by total synthesis. This molecule contains a sensitive dihydrobisfuran, functionally a masked dialdehyde, and a partially methylated hydroquinone. These reactive structural elements were set in place in good overall yield using a general method for the synthesis of highly substituted xanthones recently developed in this laboratory (Schemes 4−6).[13] Specifically, conversion of an arylnitrile to its *N*-isopropylnitrilium salt with $SbCl_5$ and 2-chloropropane followed by Houben−Hoesch reaction with an appropriately substituted aryl partner proceeded in high yield to a xanthone intermediate, whose carbonyl was protected as a butylene by addition of *n*-butyllithium. With the xanthone nucleus stabilized, the skeleton of the dihydrobisfuran was constructed, and the carbonyl was then restored by exceedingly facile peracid oxidation. With a sample of HOMST (**6**) in hand, a yeast membrane preparation containing expressed OrdA was found to convert OMST (**3**, R = R′ = Me) to AFB_1 (**4**) and to accumulate transient amounts of HOMST as secured by chromatographic comparisons and MALDI-MS. Similarly, HOMST could be independently converted to AFB_1 in parallel with OMST itself. As anticipated, the tetrahydrobisfuran-containing analogues of these metabolites **32** and **34**, respectively, were converted to AFB_2 (**33**, Scheme 7).

For quite some time a consensus view of the cytochrome P-450 hydroxylation reaction has invoked hydrogen abstraction from substrate by a heme iron-oxo species and "rebound" to product by recombination of the alkyl radical and iron-bound

Scheme 7



Scheme 8



hydroxyl radical (Scheme 8). Recent experiments with radical clocks and the intervention of cationic rearrangements, however, have complicated the mechanistic interpretation of these oxidations.[45,46] In parallel to the electrophilic mechanisms that have been advanced to account for these findings, evidence has been gathered to suggest that aldehydes can undergo nucleophilic reaction with iron-peroxy intermediates to lead on to deformylation reactions during product formation.[47−49]

The overall mechanism suggested above in Scheme 2 hypothesizes two electrophilic reactions (epoxidation) mediated by OrdA to cleave the xanthone A-ring. C-11 is ultimately lost in a subsequent ionic step in this mechanism as carbon dioxide. The demonstrated intermediacy of HOMST (**6**) limits the possible mechanisms of the second oxidation to initiate the cleavage and rearrangement to AFB_1 (cf. Scheme 2). One might object to the necessity in Scheme 2 to epoxidation in two regiochemical senses in the A-ring to achieve proper ^{18}O labeling and loss of C-11. An alternative suggestion can be advanced (but unfortunately not proved) that, after the first electrophilic oxidation to **5** and NIH shift to HOMST (**6**), nucleophilic attack by a iron-peroxy species **35** on the keto tautomer of **6** would involve little motion of the substrate relative to the iron center and lead directly to Baeyer−Villiger-like rearrangement to the seven-membered lactone **9** (Scheme 9). Moreover, by avoiding electrophilic reaction to an epoxide, the propensity to quinone formation can be circumvented. Opening of the lactone by water present in the active site, or by ferric hydroxyl/water formed in the reaction, or conceivably by an enzyme X-group to **37** (X = OH, X) can be visualized. Decarboxylation yields a stabilized anion, which can close to **38**. β-Elimination from the enol would afford **39**, which upon loss of methanol from the labile vinyl ether can be hypothesized

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(44) Yabe, K.; Ando, Y.; Hamasaki, T. *Agric. Biol. Chem.* **1991**, *55*, 1907−1911.
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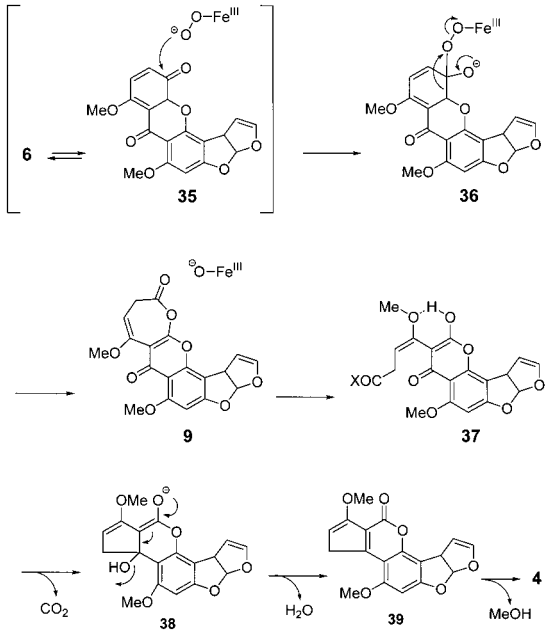
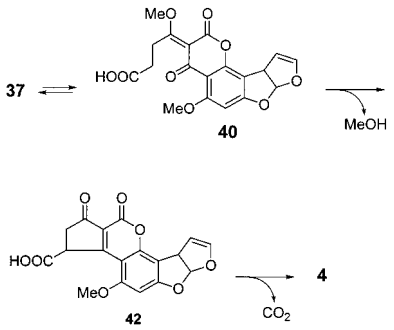
(49) Kuo, C.-L.; Raner, G. M.; Vaz, A. D. N.; Coon, M. J. *Biochemistry* **1999**, *38*, 10511−10518.

Scheme 9



Scheme 10



to give AFB_1 (**4**). In the steps past lactone **9** we invoked spontaneous chemical reactions. Interestingly, sterigmatocystin (**3**, R = Me, R′ = OH) lacking the A-ring methoxy group is not a substrate for the enzyme. It may be that the presence of this methoxy group enforces the hydroquinone oxidation state in ring-A and maintains the syn-geometry in hypothetical intermediate **37** to favor five-membered ring formation in the proposed decarboxylative aldol reaction.

Alternatively, the last step after lactone **9** could take place in a quite different manner in which tautomerization of **37** would give the stabilized **40** (a doubly vinylogous ester, Scheme 10), where anion formation adjacent to the carboxylate and intramolecular condensation with loss of water and methanol would afford **42**. Here decarboxylation to **4** would be expected to be especially facile to give an anion vinylogously delocalized to both the ketone and the lactone. These speculations await more detailed examination at the level of purified enzyme to further dissect the mechanism of this unusual P-450-mediated transformation.

Experimental Section

General Materials and Methods. Melting points were determined with a Thomas−Hoover oil bath apparatus in open capillaries and are uncorrected. ^1H and ^{13}C NMR spectra were recorded on a Bruker AMX-300 (300 MHz) or Varian Unity^{Plus} 400 MHz spectrometer and are referenced to $CDCl_3$ (7.26 and 77.0 ppm), acetone-d_6 (2.04 and 29.9 ppm), or CD_2Cl_2 (5.32 and 54.0 ppm) as indicated by the individual experiments. High- and low-resolution mass spectra were recorded using a VG Instruments 70-S 250 GC/MS at 70 eV in EI^+, CI^+, or FAB operating modes. MALDI-TOF data were obtained on a Bruker BIFLEX III. 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 on EM silica gel 60 (230−400 mesh). A ratio of 25−100:1 silica gel/crude product by weight and flow rates of 1−2 in/min were normally employed for flash columns. Thin-layer chromatography was performed on Analtech Uniplate glass plates containing fluorescent indicator, visualized by UV and 20% ethanolic solution of phosphomolybdic acid reagent and photographed with a Kodak DC290 digital camera, or by film photography by Johns Hopkins University staff. Reagents and AFB_1 and AFB_2 standards were supplied by Aldrich Chemical Co. (Milwaukee, WI), and all media components were from Difco (Detroit, MI) or Fisher (Pittsburgh, PA). All nonaqueous reactions were performed in flame-dried glassware under a positive atmosphere of dry N_2 or Ar. All solvents were dried and distilled immediately prior to use (THF and Et_2O were distilled from sodium/benzophenone, and CH_2Cl_2 and CH_3CN were distilled from CaH_2). HPLC was performed with a Perkin-Elmer 235C diode array detector and Series 410 LC pump using a Spherex 5 C18 analytical column from Phenomenex (Torrance, CA). PCR primers were purchased from Sigma-Genosys (The Woodlands, TX), and sequencing reactions were performed at the Biosynthesis and Sequencing Facility at the Johns Hopkins Medical Institute. The pYES2 yeast expression vector was purchased from Invitrogen (Carlsbad, CA), while the pT7Blue vector and Perfectly Blunt cloning kit were purchased from Novagen (Madison, WI). All restriction enzymes were from Stratagene (La Jolla, CA) or Life Technologies (Grand Island, NY). DNA, mRNA, and PCR products were purified using kits purchased from Qiagen (Valencia, CA).

Fungal and Yeast Strains and Media. The *A. parasiticus* wild-type strain SU-1 (ATCC 56775) and the ST producer *A. versicolor* (ATCC 28286) were purchased from the American Type Culture Collection (Manassas, VA). *A. parasiticus* and *A. versicolor* strains were plated on potato dextrose agar (PDA) plates,[5] which contained yeast extract, 2.5 g; Bacto-agar, 2.5 g; potato dextrose agar, 19 g; and distilled water. Liquid cultures of *A. parasiticus* (500 mL) were grown in Adye and Mateles (AM) growth medium,[50] which contained per L: sucrose, 50 g; potassium phosphate monobasic, 10 g; ammonium sulfate, 3 g; magnesium sulfate (anhydrous), 1 g; and trace metals, 2 mL. *A. versicolor* strains were grown in CZAPEK medium,[5] which contained per L: sucrose, 30 g; sodium nitrate, 3 g; potassium phosphate monobasic, 1 g; magnesium sulfate (anhydrous), 0.5 g; potassium chloride, 0.5 g; and ferrous sulfate, 0.01 g.

The *S. cerevisiae* strains INVSc1 and INVSc2 (Invitrogen, Carlsbad, CA) were used interchangeably as yeast expression hosts. Prior to transformation, all *S. cerevisiae* host strains were grown in YPD medium,[51] which contained per L: yeast extract, 10 g; bacto-peptone, 20 g; and dextrose, 20 g. Once transformed, the yeast were grown in a selective medium containing per L: 1.7 g of yeast nitrogen base [not containing amino acids or $(NH_4)_2SO_4$], 5 g of $(NH_4)_2SO_4$, 20 g of glucose, and 30 mg each of histidine, tryptophan, and leucine. The yeast expression medium was similar, but glucose was replaced with 20 g of galactose. Selective plates for yeast transformants consisted of growth medium supplemented with 15 g/L of bacto-agar.

Preparation of Sterigmatocystin (3, ST; R = Me, R′ = OH) and *O*-Methylsterigmatocystin (3, OMST; R = R′ = OMe). ST was purified from an *A. versicolor* liquid culture on the basis of a method

(50) Adye, J.; Mateles, R. I. *Biochim. Biophys. Acta* **1964**, *86*, 418−420.
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previously reported.[5] A fresh stock of *A. versicolor* was plated onto PDA plates and grown in the dark for 5 days until spore growth was heavy. Spores were collected by suspension in 9 mL of 0.05% Tween 80, and 4 mL was used to inoculate 4 × 1 L CZAPEK medium. The cultures were grown in the dark at 28 °C on a shaker at 175 rpm for 19 days. Cells were collected by filtration through cheesecloth, frozen in liquid nitrogen, and steeped in acetone. The flow through was pooled and extracted with chloroform. All organic extracts were pooled, filtered through Celite 545 (Fisher), and dried over anhydrous $MgSO_4$. Solvent was evaporated, the solid residue redissolved in 99:1 $CHCl_3$:MeOH, and ST was purified by silica column chromatography. OMST was then quickly and easily prepared from ST by methylation using a technique previously reported.[12]

Reverse Transcription of Fungal mRNA. Wild-type *A. parasiticus* was grown for 60 h from frozen spore stocks, and collected by filtration, frozen in liquid nitrogen, and ground to a powder. Total RNA was purifed from the cells using a Qiagen RNeasy Plant kit, followed by mRNA purification using a Qiagen Oligotex mRNA kit according to the manufacturer's instructions. Reverse transcription was achieved by combining in a microfuge tube: 5 μL of 10× MMLV RT buffer and 1 μL of RNAse Block (Stratagene, La Jolla, CA), 12 μL of 10 mM dNTPs (Applied Biosystems, Foster City, CA), 2 μL of poly-dT primer (Roche Molecular Biochemicals, Indianapolis, IN), 20 μL of mRNA, and 8.5 μL of distilled deionized water. The mixture was incubated at room temperature to allow the primer to bind for 15 min, then 1.5 μL of Stratagene MMLV reverse transcriptase was added, and the mixture was placed at 37 °C for 1 h. Following the reaction, the mixture was treated with 1 μL of RNAse (10 mg/mL) for 20 min at room temperature, and could then be used as template for PCR.

Construction of Expression Vectors. The cDNA prepared above was used in a PCR with primers DUapoK-1 (5′-GG**GGTACC**CCATGATTTATAGCATAATTATTTGT-3′; bold = *Kpn*I restriction site) and DUapoX-2 (5′-GC**TCTAGA**GCTCAAATCATCTGATTTCTGGC-3′; bold = *Xba*I restriction site). The 1.5 kb PCR product was purified with a Qiagen QIAquick gel extraction kit, and ligated into the cloning vector pT7Blue-3 using a Perfectly Blunt Cloning Kit, yielding the vector pT7CapoKX. The insert was retrieved by excision with *Kpn*I and *Xba*I and ligated into the multiple cloning site of the yeast expression vector pYES2 (Invitrogen) to construct the vector pYCapoKX. To build the expression vector for the N-terminally modified *ordA*, pT7CapoKX was used as template with a new 5′ primer, DUapoDK (5′-**GGTACC**ATGATTCTGCTGGCGCCCAAAGAC-3′; bold = *Kpn*I restriction site) and DUapoX-2. The PCR product was then cloned into pT7Blue-3, the insert was retrieved by digestion with *Kpn*I and *Xba*I, and ligated into pYES2, yielding the yeast expression vector pYCapoDKX.

Expression of ordA in Yeast. Yeast transformation was achieved using a lithium acetate transformation protocol,[52] and was plated onto selective medium plates. Yeast transformants were picked from plates, and a seed culture was grown to saturation ($A_{600} \approx 1.5$) in 5 mL of growth medium over 2 days at 30 °C. Two different production scales were used to express *ordA* in yeast. The small-scale expression was used to screen transformed yeast colonies, or otherwise verify *ordA* activity before large-scale expressions. In this method 500 μL of seed culture was used to inoculate 5 mL of expression medium, supplemented with 50 μg of OMST or HOMST in a minimal volume of acetone. The culture was allowed to shake 24 h at 28 °C and 300 rpm, and then was extracted with $CHCl_3$ and analyzed by TLC for aflatoxin production. For large-scale expressions 100 mL of expression media was inoculated with 1 mL of seed culture, fed with 1 mg of OMST or HOMST in a minimal volume of acetone, and allowed to shake for 24−48 h at 28 °C and at 300 rpm before extraction with $CHCl_3$ and analysis by HPLC, TLC, or MS.

Disruption of Yeast Cells. One hundred milliliter cultures of *ordA*-expressing yeast and untransformed yeast were grown to saturation in expression medium (defined above). The cells were collected by centrifugation, washed with water, and resuspended in a buffer consisting of 30% glycerol, 50 mM K_xPO_4 pH 7.4, 100 μM benzamidine, and 1 mM EDTA. After disruption with glass beads using a standard method[51] the lysed cells were centrifuged 1 h at 12 000*g*, the supernatant was decanted and saved, and the membrane pellet was washed once and resuspended in buffer.

Synthesis. Full experimental details for intermediates and reagents shown in Schemes 4−6 not described below can be found in the Supporting Information.

2-Fluoro-6-methoxy-3-pivaloylbenzonitrile (18). To a suspension of 2-fluoro-3-hydroxy-6-methoxybenzonitrile (5.63 g, 33.71 mmol, see Supporting Information) in CH_2Cl_2 (170 mL, 0.2 M) under argon was added pivaloyl chloride (6.6 mL, 53.94 mmol) and pyridine (4.9 mL, 60.68 mmol). The resulting solution was stirred 2 h and then quenched by the addition of saturated NH_4Cl. The mixture was extracted with saturated $CuSO_4$, cold 5% HCl, 5% $NaHCO_3$, and brine. The organic portion was dried ($MgSO_4$), filtered, and concentrated to an orange oil, which was triturated with ether and hexane to provide 3 g of pure product. The mother liquor was purified by column chromatography (10 → 30% EtOAc/hexanes) to give an additional 4.86 g (93% total yield) of **18**, mp 73−74°. $R_f = 0.45$ (20% EtOAc/hexane). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.25 (dd, $J = 9.2$, 8.4 Hz, 1H), 6.72 (dd, $J = 9.2$, 1.4 Hz, 1H), 3.90 (s, 3H), 1.32 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 175.9, 159.3 (d, 3.6), 155.3 (d, 259.7), 131.9 (d, 11.4), 129.0 (d, 3.2), 110.7, 106.6 (d, 3.7), 92.6 (d, 15.4), 56.7, 56.6, 39.0, 26.9. IR ($CHCl_3$): 3018, 2972, 2237, 1760, 1493, 1254, 1103 cm^{-1}. MS (EI) *m*/*e* (rel inten): 251 (M^+, 5), 167 (100), 152 (9). Anal. Calcd for $C_{13}H_{14}FNO_3$: C, 62.15; H, 5.58. Found: C, 62.14; H, 5.56.

2,4-Dihydroxy-6,6′-dimethoxy-2′-fluoro-3-methoxycarbonyl-3′-pivaloyl-benzophenone-*N*-(2-methylethyl)ketimine (21). To a CH_2Cl_2 solution (460 mL, 0.06 M) of benzonitrile (**18**, 7.0 g, 27.89 mmol) and 2-chloropropane (25.5 mL, 278.89 mmol) was added $SbCl_5$ (27.9 mL, 36.26 mmol, 1 M in CH_2Cl_2) over 8 min. After 40 min, methyl 2,6-dihydroxy-4-methoxybenzoate (**14**, 13.81 g, 69.72 mmol) was added in 80 mL of CH_2Cl_2 by cannula, and the reaction mixture was stirred for 2 h at room temperature. The volume was reduced to 200 mL, and the reaction mixture was filtered through a short silica pad and washed with 5% CH_3OH/CH_2Cl_2 to remove the antimony salts. Column chromatography (40% → 60% EtOAc/hexanes → 20% CH_3CN/EtOAc) provided 12.98 g (95%) of **21** as a yellow oil. $R_f = 0.6$ (2%MeOH/CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): δ 17.00 (brs, 1H), 13.22 (s, 1H), 7.08 (dd, $J = 8.8$, 8.4 Hz, 1H), 6.69 (dd, $J = 9.2$, 1.2 Hz, 1H), 5.54 (s, 1H), 4.00 (s, 3H), 3.75 (s, 3H), 3.46 (m, 1H), 3.36 (s, 3H), 1.35 (s, 9H), 1.23 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 178.2, 176.3, 173.3, 172.5, 166.5, 160.8, 153.5 (d, 6.4), 150.0 (d, 247.0), 132.3 (d, 13.7), 123.7, 114.5 (d, 17.8), 105.6 (d, 3.2), 101.6, 98.1, 88.0, 56.2, 55.4, 52.2, 48.3, 39.0, 27.0, 23.3, 23.0. IR ($CHCl_3$): 3032, 2984, 1752, 1630, 1596, 1571, 1550, 1500, 1450, 1109, 1089 cm^{-1}. MS (EI) *m*/*e* (rel inten): 491 (M^+, 40), 459 (100), 428 (13), 375 (15), 360 (15), 298 (17). Anal. Calcd for $C_{25}H_{30}FNO_8$: C, 61.10; H, 6.11. Found: C, 59.12; H, 6.16.

3,5-Dihydroxy-1,8-dimethoxy-4-methoxycarbonyl-9*H*-xanthen-9-one (12). A suspension of **21** (1 g, 2.04 mmol), K_2CO_3 (5.6 g, 40.36 mmol), KF·Al_2O_3[18] (0.33 g, 2.04 mmol), and 18-crown-6 (0.05 g, 0.2 mmol) in 200 mL of dry CH_3CN was heated to reflux and stirred for 6 h. A 1:1 mixture of CH_3OH/H_2O (200 mL) was added, and the resulting solution was stirred at reflux for 1 h, then overnight at room temperature. The reaction mixture was concentrated to an orange residue and dissolved in 100 mL of 1 N HCl and 300 mL of CH_2Cl_2, and extracted 3× with 100 mL portions of CH_2Cl_2. The organic extracts were dried ($MgSO_4$), filtered, and concentrated under vacuum. Purification by flash chromatography (2.5% CH_3OH/10% CH_3CN/CH_2Cl_2) afforded 0.53 g (75%) of **12** as a white solid, mp 244−245 °C (decomp). $R_f = 0.2$ (2% MeOH/CH_2Cl_2). ^{1}H NMR (300 MHz, $CDCl_3$): δ 12.39

(52) Chen, D. C.; Yang, B. C.; Kuo, T. T. *Curr. Genet.* **1992**, *21*, 83−84.

(s, 1H), 7.18 (d, J = 8.9 Hz, 1H), 6.70 (d, J = 8.9 Hz, 1H), 6.38 (s, 1H), 5.81 (brs, 1H), 4.10 (s, 3H), 3.99 (s, 3H), 3.92 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6/pyridine-d_5): δ 173.6, 168.1, 164.7, 163.7, 156.7, 151.6, 145.3, 139.8, 119.7, 114.4, 107.2, 106.7, 98.1, 95.3, 56.2, 55.8, 52.0. IR ($CHCl_3$): 3534, 3009, 2934, 1652, 1582, 1483, 1291, 1263 cm^{-1}. MS (EI) *m*/*e* (rel inten): 346 (M^+, 75), 314 (100), 299 (78), 285 (13). Anal. Calcd for $C_{17}H_{14}O_8$: C, 58.96; H, 4.05. Found: C, 58.72; H, 4.08.

1,8-Dimethoxy-5-(*t*-butyldimethylsilyloxy)-3-hydroxy-4-methoxycarbonyl-9*H*-xanthen-9-one (22). To a suspension of xanthone **12** (1.74 g, 5.03 mmol) in CH_2Cl_2/CH_3CN (3:2, 125 mL, 0.04 M) was added TBDMSCl (2.27 g, 15.09 mmol) and imidazole (2 g, 29.1 mmol). After 1 h the reaction mixture was quenched by the addition of cold 5% HCl and extracted with 5% $NaHCO_3$ and brine. The dried ($MgSO_4$) filtrate was concentrated to ca. 15 mL of CH_3CN and treated with 20 drops of 49% aqueous HF. After 30 min, the reaction mixture contained only the mono-TBS product. The mixture was diluted with CH_2Cl_2 and extracted with 5% $NaHCO_3$ and brine. After drying ($MgSO_4$) and filtering, the organic portion was concentrated and purified by column chromatography (2.5% CH_3OH/10% CH_3CN/CH_2Cl_2) to provide 1.77 g (77%) of the product as a tan solid. Recrystallization from hot EtOAc/CH_2Cl_2 and hexane afforded **22** as colorless crystals, mp 202−203 °C. R_f = 0.39 (2.5% CH_3OH/10% CH_3CN/CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): δ 12.24 (s, 1H), 7.08 (d, J = 8.8 Hz, 1H), 6.63 (d, J = 9.2 Hz, 1H), 6.35 (s, 1H), 4.02 (s, 3H), 3.96 (s, 3H), 3.90 (s, 3H), 0.97 (s, 9H), 0.21 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.9, 170.5, 167.7, 165.3, 158.0, 153.7, 147.8, 137.5, 124.0, 114.7, 108.2, 105.8, 95.6, 95.0, 56.5, 53.1, 23.8, 18.7, −3.9. IR ($CHCl_3$): 3006, 2955, 2925, 2854, 1652, 1581, 1490, 1333, 1111, 1075 cm^{-1}. MS (EI) *m*/*e* (rel inten): 460 (M^+, 11), 403 (100), 373 (77). Anal. Calcd for $C_{23}H_{28}O_8Si$: C, 60.00; H, 6.09. Found: C, 59.83; H, 6.16.

1,8-Dimethoxy-5-(*t*-butyldimethylsilyloxy)-4-methoxycarbonyl-3-*O*-methoxymethyl-9*H*-xanthene-$\Delta^{9,\delta}$-butane (24). To a −82 °C THF solution (50 mL, 0.04 M) of protected xanthone **23** (0.96 g, 1.91 mmol) was added 1.9 mL of *n*-BuLi (2.5 mmol, 1.3 M). After 1 h, 20 mL of CH_3OH and 20 mL of 1 M tartaric acid were added, and the resulting mixture was stirred for 45 min and warmed to room temperature. The mixture was diluted with EtOAc, and the two phases were separated. The aqueous portion was back-extracted with $CHCl_3$ until the aqueous layer was colorless, and the combined organic layers were washed with 5% $NaHCO_3$ and brine. After drying (MgSO), filtering, and concentrating to an orange oil, the residue was loaded onto a silica gel column and eluted with three column volumes of 1% HOAc/$CHCl_3$ to facilitate the dehydration of the tertiary alcohol. Finally, eluting with 1% HOAc/5% acetone/$CHCl_3$ provided the pure **24** as a yellow oil (0.95 g, 92%), as a mixture of (*E*)- and (*Z*)-isomers. R_f = 0.4 (20% EtOAc/hexane). ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.75 (d, J = 8.9 Hz, 1H), 6.64 (d, J = 8.9 Hz, 1H), 6.53 (s, 1H), 6.52 (s, 1H), 6.52 (d, J = 8.8 Hz, 2H), 6.18 (m, 1H), 6.09 (m, 1H), 5.20 (s, 2H), 5.17 (s, 2H), 3.93 (s, 3H), 3.92 (s, 3H), 3.85 (s, 6H), 3.79 (s, 6H), 3.49 (s, 3H), 3.47 (s, 3H), 2.25 (m, 2H), 1.81 (m, 2H), 1.38 (m, 4H), 1.01 (s, 18H), 0.89 (t, J = 7.3 Hz, 3H), 0.85 (t, J = 7.3 Hz, 3H), 0.24 (s, 6H), 0.19 (s, 6). ^{13}C NMR (100 MHz, $CDCl_3$): δ 165.6, 157.1, 157.0, 154.9, 153.6, 152.7, 151.2, 149.6, 149.4, 145.6, 144.3, 137.8, 137.5, 133.9, 133.8, 119.0, 118.3, 118.1, 118.0, 117.6, 115.5, 111.7, 108.8, 107.0, 106.7, 106.0, 105.4, 95.2, 95.1, 94.8, 93.9, 56.3, 56.0, 55.9, 55.5, 55.4, 52.4, 33.3, 33.1, 25.9, 22.7, 18.4, 14.1, 13.9, −4.3, −4.5. IR ($CHCl_3$): 3006, 2956, 2847, 1730, 1575, 1495, 1252, 1084, 1049 cm^{-1}. MS (EI) *m*/*e* (rel inten): 544 (M^+, 15), 487 (100), 447 (15), 441 (31). HRMS (EI) calcd for $C_{29}H_{40}O_8Si$ (M^+), 544.2492; found, 544.2498.

5-(*t*-Butyldimethylsilyloxy)-1,8-dimethoxy-4-(ethyl-3′-formylpropanoate)-3-*O*-methoxymethyl-9*H*-xanthen-$\Delta^{9,\delta}$-butane (26). To a cooled (−78 °C) solution of 1-(*N*-benzylideneamino)aminomethylphosphonate (0.66 g, 2.9 mmol) in 15 mL of dry THF was added *n*-butyllithium (2.0 mL, 1.47 M in hexanes, 2.9 mmol) over 5 min. This solution was stirred at −78 °C for 1 h. A solution of aldehyde **25** (1.25 g, 2.43 mmol) in 6 mL of dry THF was added to the resulting lithium benziminomethyl-phosphonate[53,54] by cannula over 5 min. After 50 min, the mixture was allowed to stir at room temperature for 1.5 h and then was cooled to −78 °C, and *n*BuLi (2.32 mL, 1.47 M, 3.41 mmol) was added over 3 min. After 2.5 h, bromoethyl acetate (1.35 mL, 12.17 mmol) was added to the −78 °C mixture, which was then allowed to warm to room temperature over 1.5 h. The reaction was quenched by the addition of 7 mL of 1 M aqueous tartaric acid and stirred for 1 h. The mixture was extracted with cold 5% HCl (2×), 5% $NaHCO_3$ (2×), and brine, dried ($MgSO_4$), filtered, and concentrated under vacuum. The residue was purified by flash chromatography (2 ⟹ 40% EtOAc/hexane) to give 1.18 g (79%) of **26** as a yellow oil and a mixture of *E* and *Z* isomers. R_f = 0.39 (20% EtOAc/hexane). ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.72 (s), 9.69 (s, 1H), 9.62 (s, 1H), 6.77 (d, J = 8.8 Hz, 1H), 6.66 (d, J = 8.8 Hz, 1H), 6.58 (s, 1H), 6.55 (s, 1H), 6.52 (d, J = 8.8 Hz, 2H), 6.20 (m, 1H), 6.10 (m, 1H), 5.14 (m, 4H), 4.10 (q, J = 7.2 Hz, 4H), 4.06 (m, 2H), 3.84 (s, 6H), 3.79 (s, 6H), 3.45 (s, 3H), 3.43 (s, 3H), 3.24 (m, 2H), 2.69 (m, 2H), 2.30 (m, 2H), 1.86 (m, 2H), 1.45 (m, 4H), 1.24 (t, J = 7.1 Hz, 6H), 0.99 (s, 18H), 0.88 (t, J = 7.3 Hz, 6H), 0.30 (s, 3H), 0.28 (s, 3H), 0.23 (s, 3H), 0.22 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 199.8, 199.7, 171.9, 171.7, 155.9, 155.8, 155.7, 154.8, 154.6, 153.9, 152.5, 152.3, 149.5, 149.4, 145.6, 144.3, 137.8, 137.6, 137.4, 133.6, 133.5, 118.8, 118.6, 118.5, 118.3, 117.3, 117.0, 116.1, 115.6, 111.6, 108.7, 105.9, 105.6, 105.3, 105.2, 94.5, 94.4, 94.0, 93.1, 60.2, 56.2, 56.1, 55.9, 55.7, 55.2, 44.2, 33.2, 33.0, 32.9, 32.8, 25.8, 22.5, 18.3, 14.0, 13.8, −4.1, −4.2, −4.4. IR ($CHCl_3$): 3012, 2958, 2922, 2851, 1724, 1573, 1492, 1465, 1256, 1099, 1055 cm^{-1}. MS (EI) *m*/*e* (rel inten): 614 (M^+, 48), 583 (48), 557 (100). HRMS (EI) calcd for $C_{33}H_{46}O_9Si$ (M^+), 614.2911; found, 614.2918.

10-(*t*-Butyldimethylsilyloxy)-5,7-dimethoxy-1-(ethoxycarbonylmethyl)-2-[tris(1-methylethyl)silyloxy)]-6*H*-furo[2,3-*c*]xanthene-$\Delta^{6,\delta}$-butane (27). Aldehyde **26** (1.03 g, 1.67 mmol) and triethylamine (0.37 mL, 2.68 mmol) were combined in 21 mL of dry THF and cooled to 0 °C. Triisopropylsilyl trifluoromethylsulfonate (0.54 mL, 2.0 mmol) was added dropwise, and the mixture was stirred for 3 h and warmed to room temperature. The reaction was quenched by the addition of *N*,*N*-dimethylethanolamine (0.5 mL, 5.02 mmol) and extracted with cold 5% HCl, 5% $NaHCO_3$, brine, and dried ($MgSO_4$), filtered, and concentrated under vacuum. The residue was purified by flash chromatography (5% ethyl acetate/hexane) to provide 1.06 g of **27** (87%) as a colorless oil, which was a mixture of four isomers: (*E*)- and (*Z*)- and *cis*- and *trans*-acetals, mp 51−54 °C. R_f = 0.55 (10% EtOAc/hexane). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.78 (d, J = 8.8 Hz, 1H), 6.67 (d, J = 8.8 Hz, 1H), 6.54 (d, J = 8.8 Hz, 2H), 6.29 (s, 1H), 6.27 (s, 1H), 6.15 (m, 2H), 5.93 (m, 2H), 4.14 (m, 4H), 3.83 (s, 6H), 3.81 (s, 6H), 3.76 (m, 4H), 3.12 (ddd, J = 19.6, 16.8, 3.2 Hz, 1H), 2.99 (dd, J = 16.8, 2.8 Hz, 1H), 2.47 (m, 2H), 2.31 (m, 2H), 1.89 (m, 2H), 1.44 (m, 4H), 1.27 (t, J = 7.2 Hz, 3H), 1.22 (t, J = 7.2 Hz, 3H), 1.09 (m, 42H), 1.05 (s, 9H), 1.04 (s, 9H), 0.90 (m, 6H), 0.30 (s, 3H), 0.25 (s, 3H), 0.24 (s, 3H), 0.20 (s, 3H). ^{13}C NMR (400 MHz, $CDCl_3$): δ 171.5, 171.3, 159.3, 159.1, 158.0, 157.9, 157.0, 156.8, 156.7, 151.8, 151.6, 150.3, 149.9, 149.7, 146.0, 145.7, 144.7, 144.4, 137.8, 137.5, 137.2, 132.9, 132.8, 132.7, 132.6, 119.0, 118.9, 118.8, 118.5, 117.6, 117.2, 116.2, 110.4, 107.5, 106.9, 106.8, 106.6, 106.0, 105.9, 105.8, 105.7, 105.2, 105.1, 90.6, 90.4, 89.9, 89.6, 60.4, 56.0, 55.9, 55.5, 55.4, 46.2, 46.1, 45.9, 45.8, 36.0, 35.9, 35.2, 35.1, 33.4, 33.3, 33.2, 25.7, 22.7, 18.2, 18.1, 17.8, 17.7, 14.1, 14.0, 13.9, 12.1, −4.3, −4.4, −4.5, −4.7. IR ($CHCl_3$): 3006, 2950, 2857, 1726, 1633, 1605, 1488, 1460, 1090 cm^{-1}. MS (EI) *m*/*e* (rel inten): 726 (M+, 52), 695 (37), 669 (100). Anal. Calcd for $C_{40}H_{62}O_8Si_2$: C, 66.12; H, 8.54. Found: C, 66.10; H, 8.57.

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1-(Acetaldehyde)-10-(*t*-butyldimethylsilyoxy)-5,7-dimethoxy-2-[tris(1-methylethyl)silyloxy)]-6*H*-furo[2,3-c]xanthen-6-one (29). To a 3 mL CH_2Cl_2 solution (0.01 M) of butylene aldehyde **28** (0.18 g, 0.26 mmol) and 4 Å molecular sieves (0.02) was added ~70% *m*-CPBA (0.02 g, 0.10 mmol). After 10 min the reaction was quenched by the addition of saturated $NaHCO_3$ (2 mL) and diluted with CH_2Cl_2. The CH_2Cl_2 layer was separated and washed with brine, dried ($MgSO_4$), filtered, and concentrated to a yellow solid. Column chromatography (2.5% CH_3OH/10% CH_3CN/CH_2Cl_2) provided 0.15 g (88%) of the desired **29** as a white solid, which was recrystallized from hot EtOAc/CH_2Cl_2 and hexane, mp 172−174 °C. R_f = 0.62 (2.5% CH_3OH/10% CH_3CN/CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.81 (s, 1H), 7.00 (d, J = 9.2 Hz, 1H), 6.59 (d, J = 9.2 Hz, 1H), 6.31 (s, 1H), 5.85 (s, 1H2), 3.90 (s, 3H), 3.88 (s, 3H), 3.84 (dd, J = 11 Hz, 1H), 3.12 (dd, J = 18, 3.2 Hz, 1H), 2.63 (dd, J = 18, 11 Hz, 1H), 1.05 (m, 21H, TIPS), 0.99 (s, 9H), 0.20 (s, 3H), 0.19 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 199.9, 175.4, 163.3, 162.9, 154.0, 153.5, 147.8, 137.0, 123.0, 115.9, 108.6, 106.8, 105.6, 105.3, 90.6, 56.6, 56.4, 44.6, 43.7, 25.5, 10.1, 17.7, 17.6, 11.9, −4.4, −4.6. IR ($CHCl_3$): 3002, 2940, 2868, 1721, 1654, 1597, 1577, 1484, 1091 cm^{-1}. MS (EI) *m*/*e* (rel inten): 642 (M^+, 100), 627 (3), 599 (15), 440 (51), 411 (21). Anal. Calcd for $C_{34}H_{50}O_8Si_2$: C, 63.55; H, 7.79. Found: C, 63.39; H, 7.79.

2,11-Dihydroxy-6,8-dimethoxy-7*H*-1,2-dihydrofuro[3′,2′:4,5]furo-[2,3-*c*]xanthen-7-one (30). TEA·$(HF)_3$ (0.25 mL, 1.56 mmol) was added to a suspension of aldehyde **29** (0.090 g, 0.14 mmol) in CH_3CN (8 mL, 0.02 M). The reaction mixture clarified somewhat over the period of 1 h, at which point it was filtered through a small pad of silica and washed with copious amounts of 5% CH_3OH/CH_2Cl_2 to provide 0.038 g (73%) of **30** as a white solid, mp 253 °C (dec). R_f = 0.13 (5% CH_3OH/CH_2Cl_2). ^{1}H NMR (300 MHz, DMSO-d_6; a complex mixture of open and closed exo and endo forms giving the following signals): δ 9.15 (s, 1H), 9.08 (s), 9.04 (s), 7.07 (d), 7.06 (d, J = 8 Hz, 1H), 6.88 (d), 6.79 (d), 6.59 (d, J = 8.7 Hz, 1H), 6.41 (d, J = 6 Hz, 1H), 6.39 (d), 6.30 (s), 6.27 (s, 1H), 6.20 (s), 6.16 (m), 5.85 (m, 1H), 5.60 (m), 5.42 (m), 4.22 (m, 1H), 3.82 (s, 3H), 3.78 (s, 3H), 2.33 (m), 2.10 (m), 1.93 (d, J = 12.3 Hz, 1H). IR ($CHCl_3$): 3296, 3004, 2979, 2866, 1730, 1643, 1590, 1484, 1098 cm^{-1}. MS (EI) *m*/*e* (rel inten): 372 (M^+, 41), 354 (100), 339 (53), 325 (42). HRMS (EI) calcd for $C_{19}H_{16}O_8$ (M^+), 372.0845; found, 372.0850.

6,8-Dimethoxy-11-hydroxy-2-thiophenyl-7*H*-1,2-dihydrofuro-[3′,2′:4,5]furo[2,3-*c*]xanthen-7-one (*endo*-31). A suspension of hemiacetal **30** (0.05 g, 0.13 mmol), thiophenol (0.1 mL, 0.97 mmol), 4 Å molecular sieves (0.1 g), and Amberlyst 15 resin (0.05 g) was heated to reflux in 10 mL of CH_3CN (0.01 M) for 2 h. The resulting solution was concentrated to 3 mL, filtered, and then purified by column chromatography (20 → 30% CH_3CN/CH_2Cl_2) to provide 0.062 g (93%) of an inseparable mixture of *endo*- and *exo*-sulfides as a yellow solid, mp 273 °C (dec). R_f = 0.41 (5% CH_3OH/CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.51 (dd, J = 8, 1.6 Hz, 1H, H_{para}), 7.41 (dd, J = 8, 1.6 Hz, 2H, H_{ortho}), 7.29 (m, 2H, H_{meta}), 7.19 (d, J = 8.8 Hz, 1H, H-10), 6.70 (d, J = 8.8 Hz, 1H, H-9), 6.56 (d, J = 6 Hz, 1H, H-3a), 6.39 (s, 1H, H-5), 5.81 (d, J = 7.2 Hz, 1H, H-2), 5.14 (s, 1H, O*H*), 4.32 (dd, J = 8.4, 6 Hz, 1H, H-12c), 3.95 (s, 3H, OCH_3), 3.92 (s, 3H, OCH_3), 2.88 (m, 1H, H-1_a), 2.60 (d, J = 13.6 Hz, 1H, H-1_b). ^{13}C NMR (100 MHz, $CDCl_3$): δ endo/exo, 173.8, 173.5, 173.4, 163.9, 163.4, 162.3, 162.1, 160.3, 160.2, 156.7, 155.7, 153.2, 152.9, 152.7, 151.5, 151.4, 144.7, 144.6, 139.1, 139.0, 135.5, 134.9, 133.5, 130.8, 130.0, 129.8, 129.2, 129.0, 128.9, 127.5, 126.7, 126.5, 119.6, 119.5, 114.1, 114.0, 113.9, 113.8, 112.5, 107.9, 107.7, 107.1, 106.8, 106.7, 106.6, 105.7, 104.8, 104.4, 100.2, 99.9, 96.2, 95.6, 94.3, 90.6, 90.2, 87.5, 85.7, 56.4, 56.3, 56.1, 43.7, 43.6, 36.3. IR ($CHCl_3$): 3591, 3176, 3011, 3007, 2939, 1639, 1657, 1594, 1578, 1487, 1083 cm^{-1}. MS (EI) *m*/*e* (rel inten): 464 (M^+, 40), 355 (100), 110 (38). HRMS (EI) calcd for $C_{25}H_{20}O_7S$ (M^+), 464.0930; found, 464.0937.

***exo*-Sulfide.** ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.51 (dd, J = 8, 1.6 Hz, 1H, H_{para}), 7.41 (dd, J = 8, 1.6 Hz, 2H, H_{ortho}), 7.29 (m, 2H, H_{meta}), 7.18 (d, J = 8.8 Hz, 1H, H-10), 6.68 (d, J = 8.8 Hz, 1H, H-9), 6.54 (d, J = 6 Hz, 1H, H-3a), 6.34 (s, 1H, H-5), 5.32 (dd, J = 10.4, 4.4 Hz, 1H, H-2), 5.14 (s, 1H, O*H*), 4.27 (m, 1H, H-12c), 3.91 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3), 2.69 (dd, J = 136, 4.4 Hz, 1H, H-1_a), 2.34 (m, 1H, H-1_b).

6,8-Dimethoxy-11-hydroxy-7*H*-furo[3′,2′:4,5]furo[2,3-*c*]xanthen-7-one, (11-Hydroxy-*O*-methylsterigmatocystin, 6). To a −45 °C CH_2Cl_2 suspension (4 mL, 0.01 M) of phenylsulfides **31** (0.017 g, 0.037 mmol) was added 70% dry *m*-CPBA (0.11 g, 0.064 mmol). After 40 min, dimethyl sulfide (0.01 mL) and 10% Na_2SO_3 (2 mL) were added, and the mixture was warmed to room temperature. The resulting biphasic mixture was separated, and the aqueous portion was back-extracted twice with CH_2Cl_2. The combined organic layers were extracted with saturated $NaHCO_3$ and brine, dried over $K_2CO_3/MgSO_4$ (1:1), filtered (washing solids well with 5% CH_3OH/CH_2Cl_2), and concentrated to a tan solid. Purification by column chromatography (5% CH_3OH/CH_2Cl_2) provided the sulfoxides as a mixture of four diastereomers, which were pyrolyzed directly. The sulfoxides and diisopropylethylamine (two drops) were dissolved in toluene (3.5 mL) and DMSO (0.75 mL), placed in a preheated oil bath at 120 °C, and stirred for 1 h. The mixture was cooled to room temperature, diluted with CH_2Cl_2, and extracted with saturated $NaHCO_3$ to remove the sulfinic acid and brine. The organic portion was dried over $K_2CO_3/MgSO_4$ (3:1), filtered, concentrated to ca. 2 mL, and loaded onto a column of silica gel. Elution with 2 → 5% CH_3OH/CH_2Cl_2 provided pure **6** (8 mg, 62% yield from the phenylsulfides) as a white solid, mp 280 °C. R_f = 0.30 (5% CH_3OH/CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$/DMSO-d_6): δ 8.54 (s, 1H), 6.70 (d, J = 8.8 Hz, 1H), 6.38 (d, J = 7.2 Hz, 1H), 6.21 (d, J = 8.8 Hz, 1H), 6.05 (t, J = 2.8 Hz, 1H), 5.98 (s, 1H), 5.15 (dd, J = 2.8, 2.4 Hz, 1H), 4.51 (dt, J = 7.2, 2.4 Hz, 1H), 3.47 (s, 3H), 3.43 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$/DMSO-d_6): δ 174.1, 161.8, 161.4, 151.8, 151.3, 144.1, 143.6, 138.2, 118.8, 113.4, 112.0, 107.4, 105.5, 105.2, 102.3, 89.3, 55.7, 55.5, 47.2. IR ($CHCl_3$): 3180, 3007, 2961, 2852, 1638, 1581, 1487, 1465, 1271, 1263, 1137, 1103, 1082, 980 cm^{-1}. UV−vis (c = 8 μg/mL, CH_3OH): λ_{max} 316 (11 800), 239 (31 800). MS (CI) *m*/*e* (rel inten): 355 ($[M + H]^+$, 28), 341 (12), 102 (56). HRMS (EI) calcd for $C_{19}H_{14}O_7$ (M+), 354.0740; found, 354.0739.

Supporting Information Available: Full experimental details for intermediates and reagents shown in Schemes 4−6 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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