# The in Vitro Conversion of Norsolorinic Acid to Aflatoxin $B_1$. An Improved Method of Cell-Free Enzyme Preparation and Stabilization

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**Abstract:** The biosynthesis of the environmental carcinogen aflatoxin $B_1$ (**13**) is initiated by the formation of a $C_6$-primer by a dedicated yeast-like fatty acid synthase. Homologation of this starter unit by a polyketide synthase gives the anthraquinone norsolorinic acid (**2**). Approximately 15 chemical steps follow from this first stable intermediate to the mycotoxin (**13**) itself. A new protocol of cell-free enzyme preparation has been developed from the fungus *Aspergillus parasiticus* which carries out all of these transformations for the first time. The key experimental step involves rapid concentration and efficient dialysis by membrane filtration to remove primary and secondary metabolites, cofactors, and small biomolecules (MW < 10 000). All enzymes of the aflatoxin biosynthetic pathway have been dramatically stabilized by this procedure, and the effects of added substrates and cofactors can be assayed against virtually no background reactions. The overall pathway from norsolorinic acid (**2**) to aflatoxin $B_1$ (**13**) has been investigated, cofactor requirements defined for each step, and a time-course run in which only versicolorin A (**9**) and sterigmatocystin (**11**) were observed to accumulate. The post-bisfuran skeletal rearrangement of versicolorin A (**9**) to demethylsterigmatocystin (**10**) was studied in *O*-methylsterigmatocystin (**12**) in the presence of $D_2O$ and/or $d_7$-glucose or stereospecifically labeled NADPD. Unexpectedly high extents of proton exchange were found in the A ring during this transformation, including at a site of formal reduction. A tentative mechanism is discussed to account for this multi enzyme process.

## Introduction

Molecular biology has revolutionized the study of enzyme structure and function. Powerful methods exist to identify genes by sequence homology, to amplify them, and to sequence very large genomic regions rapidly and accurately. For natural product biosynthetic pathways in bacteria it has been found invariably the case that their encoding genes are clustered.[1,2] This physical arrangement is of obvious advantage for the study of such pathways, and is sometimes observed in fungi as well. Unfortunately, while dramatic advances have been made to localize biosynthetic genes using similarity-based cloning strategies, as for example polyketide synthases[3] or terpene cyclases,[4] a growing number of biosynthetic gene clusters is being defined where the functions of translated proteins are not usefully revealed by examination of many, even most, open reading frames contained within them. The converse of this observation is also true that genes identified by reverse-genetic techniques from proteins with demonstrated roles in a biosynthetic pathway give no discernible correspondence to their actual function and can suggest an entirely erroneous function.[5]

Despite these difficulties, specific disruption or replacement of a given gene, gene "knockout" experiments, can lead to the accumulation of an intermediate and, thus, help define the function of the gene product. Nonetheless, this standard method can be thwarted by gene duplication or, in practice, other less well-understood processes. The most compelling evidence of enzyme function is precisely that—function. Similarly, the chemical questions of catalysis and mechanism, or modification of these proteins through mutagenesis or combinatorial means to change the products generated, rely on tests of actual function. This goal can be achieved by overexpression of a biosynthetic gene to give a correctly folded (and, as needed, correctly co- or posttranslationally modified) protein whose function can be thoroughly characterized. Unfortunately, there remain practical limitations where overproduction methods can fail or give poor yields of active protein.

Alternatively, although often not seriously pursued, biochemical techniques can afford the wild-type enzyme for direct study. We report here a significant methodological improvement in the preparation and stabilization of cell-free extracts (CFE) of the fungus *Aspergillus parasiticus* to study the biosynthesis of the potent environmental carcinogen aflatoxin $B_1$ (AFB1, **13**; Scheme 1). All steps from the initial construction of norsolorinic acid (NOR, **2**) by a pair of specialized fatty acid synthases (FAS) and a simple Type I polyketide synthase (PKS) to the mycotoxin itself have been achieved. While the FAS/PKS steps will be described elsewhere, we illustrate this new method for the NOR → $AFB_1$ part of the pathway and, in particular, focus on a set of experiments to examine the poorly understood anthraquinone to xanthone transformation represented by the conversion of versicolorin A (**9**) to demethylsterigmatocystin (**10**). We believe this method of CFE preparation will be generally useful in the study of a wide variety of hitherto

(1) Hopwood, D. A.; Sherman, D. H. *Annu. Rev. Genet.* **1990**, *24*, 37−66.

(2) Ward, J. M.; Hodgson, J. E. *FEMS Microbiol. Lett.* **1993**, *110*, 239−242.

(3) Schwecke, T.; Aparicio, J. F.; Molár, I.; König, A.; Khaw, L. E.; Haydock, S. F.; Oliynyk, M.; Caffrey, P.; Cortés, J.; Lester, J. B.; Böhm, G. A.; Staunton, J.; Leadlay, P. F. *Proc. Nat. Acad. Sci.* **1995**, *92*, 7839−7843.

(4) Steele, C. L.; Lewinsohn, E.; Croteau, R. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 4164−4168.

(5) Silva, J. C.; Minto, R. E.; Barry, C. E., III; Holland, K. A.; Townsend, C. A. *J. Biol. Chem.* **1996**, *271*, 13600−13608.

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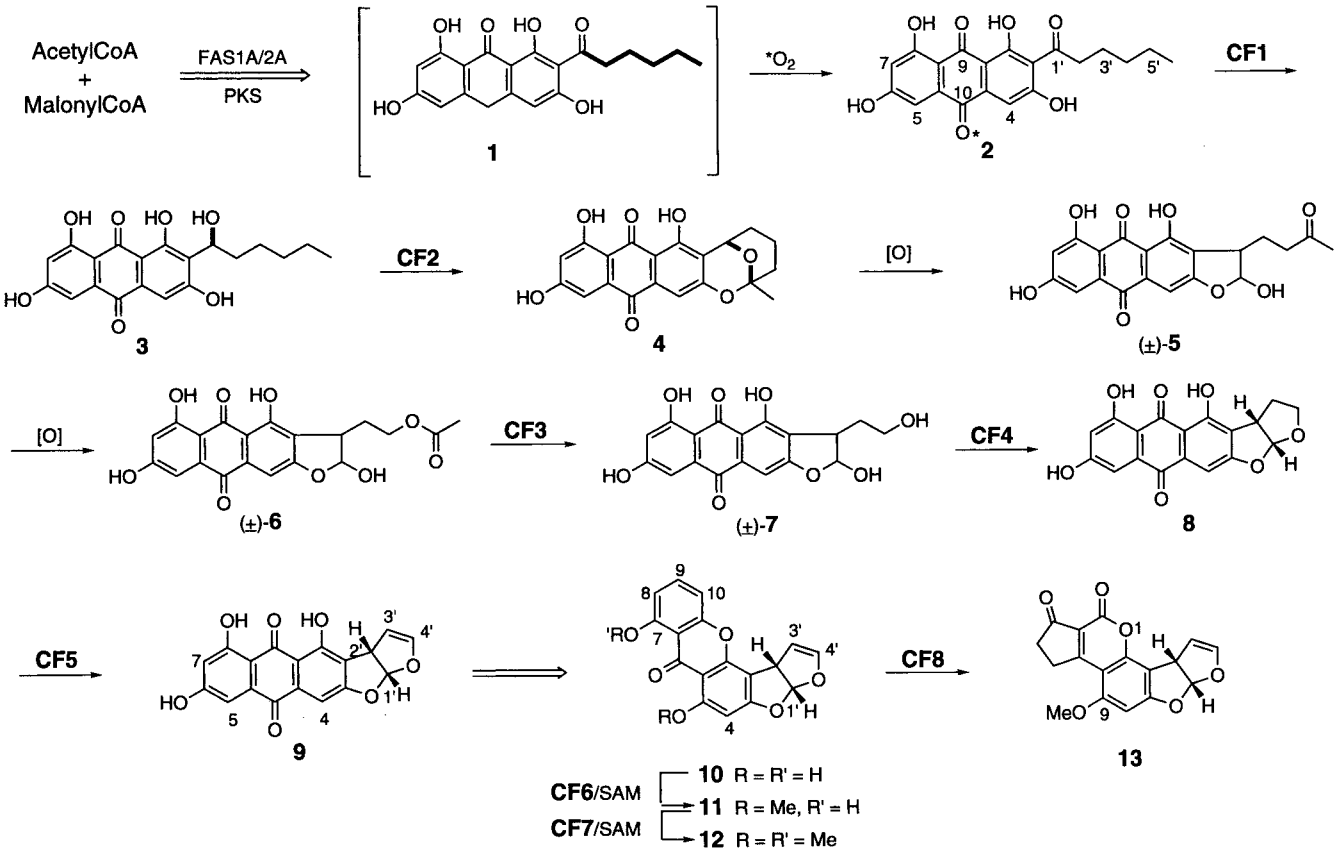
**Scheme 1**




inaccessible enzymes and provides a needed complement to gene-based methods.

The aflatoxin $B_1$ (**13**) biosynthetic pathway is unusually long and complex requiring on the order of 15 separate enzymic transformations, not including the iterative steps involved in the FAS/PKS assembly of NOR (**2**).[6] The principal intermediates are shown in Scheme 1. Dedicated effort over many years by several research groups has resulted in the observation of several individual and a few sequential transformations in the pathway by cell-free systems. These are arbitrarily denoted as CF1−8 in Scheme 1. CF1 demonstrated an NADPH requirement for the reduction of NOR (**2**) to averantin (**3**).[7] Curiously, however, disruption of the supposed corresponding gene suggests that its encoded dehydrogenase is not the major one functioning in vivo to carry out this reduction.[8] More than one group has examined the redox steps that lead to the formation of averufin (**4**) in cell-free systems shown collectively here as CF2.[7,9,10] However, the first oxidative rearrangement of the pathway in which the first furan ring is formed in (±)-hydroxyversicolorone (**5**) has eluded observation in a cell-free extract as has the subsequent Baeyer−Villiger-like reaction to (±)-versiconal acetate (**6**). An esterase has been demonstrated (CF3) by several groups to give (±)-versiconal (**7**),[11,12] which is in turn cyclized with loss of water[13] to give the optically pure tetrahydrobisfuran versicolorin B (**8**).[11,14,15] The enzyme catalyzing this intramolecular cyclization, versicolorin B synthase (VBS), has been purified[16,17] and characterized,[15] and its gene (*vbs*) has been cloned.[18] It is instructive to note that *vbs* shows high extents of sequence identity to the GMC family of flavin-dependent oxidoreductases.[19] VBS, however, neither binds FAD or FMN, nor does it catalyze a redox reaction, despite the prevalence of these reactions in the biosynthesis of aflatoxin.[6]

Yabe has shown in a microsomal system (CF5) the desaturation of versicolorin B (VB, **8**) to versicolorin A (VA, **9**).[20] The important nuclear reorganization to the xanthone demethylsterigmatocystin (DMST, **10**) has not been sucessfully demonstrated in a cell-free preparation until the present work, which will be described below. Sequential *O*-methylations occur next from *S*-adenosylmethionine (SAM) to give (CF6) sterigmatocystin (ST, **11**) and *O*-methylsterigmatocystin (OMST, **12**) (CF7).[21−23] The gene corresponding to the latter methyltrans-

(6) Minto, R. E.; Townsend, C. A. *Chem. Rev.* **1997**, *97*, 2537−2555.
(7) Yabe, K.; Nakamura, Y.; Nakajima, H.; Ando, Y.; Hamasaki, T. *Appl. Environ. Microbiol.* **1991**, *57*, 1340−1345.
(8) Cary, J. W.; Wright, M.; Bhatnagar, D.; Lee, R.; Chu, F. S. *Appl. Environ. Microbiol.* **1996**, *62*, 360−366.
(9) McCormick, S. P.; Bhatnagar, D.; Lee, L. S. *Appl. Environ. Microbiol.* **1987**, *53*, 14−16.
(10) Yabe, K.; Matsuyama, Y.; Ando, Y.; Nakajima, H.; Hamasaki, T. *Appl. Environ. Microbiol.* **1993**, *59*, 2486-2492.
(11) McGuire, S. M.; Brobst, S. W.; Graybill, T. L.; Pal, K.; Townsend, C. A. *J. Am. Chem. Soc.* **1989**, *111*, 8308−8309.
(12) Hsieh, D. P. H.; Wan, C. C.; Billington, J. A. *Mycopathologia* **1989**, *107*, 121−126.
(13) McGuire, S. M.; Townsend, C. A. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 653−656.
(14) Yabe, K.; Hamasaki, T. *Appl. Environ. Microbiol.* **1993**, *59*, 2493−2500.
(15) McGuire, S. M.; Silva, J. C.; Casillas, E. G.; Townsend, C. A. *Biochemistry* **1996**, *35*, 11470−11486.
(16) Townsend, C. A.; McGuire, S. M.; Brobst, S. W.; Graybill, T. L.; Pal, K.; Barry, C. E., III. In *Secondary-Metabolite Biosynthesis and Metabolism*; Petroski, R. J., Ed.; Plenum Press: New York, 1992; pp 141−154.
(17) Lin, B.-K.; Anderson, J. A. *Arch. Biochem. Biophys.* **1992**, *293*, 67−70.
(18) Silva, J. C.; Townsend, C. A. *J. Biol. Chem.* **1997**, *272*, 804−813.
(19) Cavener, D. R. *J. Mol. Biol.* **1992**, *223*, 811−814.
(20) Yabe, K.; Ando, Y.; Hamasaki, T. *Agric. Biol. Chem.* **1991**, *55*, 1907−1911.
(21) Keller, N. P.; Dischinger, H. C.; Bhatnagar, D.; Cleveland, T. E.; Ullah, A. H. J. *Appl. Environ. Microbiol.* **1993**, *59*, 479−484.

ferase has been cloned (*omt1*)[24,25] and played a key role in the identification of the aflatoxin gene cluster.[26] Chemical evidence[27,28] suggests that a two-step oxidative process completes the pathway in which a single NADPH-dependent P-450 monooxygenase[29] catalyzes the cleavage, rearrangement, and decarboxylation of OMST (**12**) to $AFB_1$ (**13**).[6,28] The method of cell-free preparation we describe carries out *all* of the transformations from NOR (**2**) to $AFB_1$ (**13**) in such a way that cofactor requirements in previously known and unknown transformations can be readily demonstrated. We illustrate the usefulness of the method in deuterium-labeling experiments to examine the cryptic rearrangement of VA (**9**) to DMST (**10**).

## Results and Discussion

**Cell-Free Extract Preparation**. Previous methods to prepare cell-free extracts of *A. parasiticus* have employed both mechanical disruption, and enzymatic digestion of the mycelia to observe catalytic activities. Cell breakage by mechanical means has been achieved by grinding with a mortar and pestle in the presence of sea sand,[30] homogenizing the cells with glass powder and a rotating metal loop,[31] or by freezing in liquid nitrogen, pulverizing the cells with either a Osterizer blender[22] or a "Bead-Beater" containing precooled glass powder.[16] Alternatively, digestion with lytic enzyme to remove the fungal cell wall has been used to achieve the formation of protoplasts.[32,33]

The new method of cell-free extract preparation entails, first, the generation of a crude cell-free extract by fairly traditional means, but is followed by a filtration step that efficiently removes small molecules and cofactors from the protein suspension. The latter step has been shown to not only greatly extend the lifetimes of the proteins, but also to enhance enzymatic reactivities as well. Initial cell breakage has been carried out by freezing the cells in liquid nitrogen and pulverizing them in a Waring blender containing cubes of dry ice. We have found mechanical disruption by this method to be the most efficient and reproducible means of cell breakage as grinding cells with a mortar and pestle can be highly user dependent and lack reproducibility. Use of the "Bead-Beater" in our hands has often resulted in warming or mild foaming of the extract, while disruption with a rotating metal loop has given low protein yields. Although protein lysates have been successfully prepared from *A. parasiticus* protoplasts, the method lacks practical utility. Protoplasts are difficult to generate in even modest amounts and result in very low protein levels making isolation and characterization of individual enzymes an extremely difficult undertaking.

Once the frozen cells have been ground to a fine powder using a Waring blender, the broken cells were suspended in an extraction buffer and allowed to stir at 4 °C for 2 h. The composition of the extraction buffer was found to be critical for effective stabilization of enzyme activities. The buffer contains 50 mM potassium phosphate (pH 7.5), 30% glycerol, 1 mM EDTA, 2 mM $\beta$-mercaptoethanol or DTT, 100 $\mu$M benzamidine, and 100 $\mu$M phenylmethylsulfonyl fluoride. Trial experiments have shown that Tris is detrimental to some enzymes of the pathway leading to complete loss of enzymatic activity. The inclusion of glycerol is imperative to support these activities, as well as the addition of protease inhibitors, $\beta$-mercaptoethanol (a reducing agent) and EDTA.[34] The addition of 1 mM EDTA to the extraction buffer has been demonstrated to enhance enzymatic conversions 5−10-fold, perhaps through inactivation of metal-dependent proteases or by aiding the solubility of weakly membrane bound proteins. Following 2 h incubation at 4 °C, the cell suspension was centrifuged at 16 000*g* for 20 min, and the supernatant was decanted and filtered through four layers of cheesecloth to remove any remaining cell debris. The crude cell-free extract was then throughly dialyzed with an Amicon RA 2000 apparatus equipped with a 10 000 molecular weight cutoff membrane by the gradual addition of 4 L of buffer to maintain a constant volume. The apparatus is highly efficient at removing background metabolites and low molecular weight chromophoric components (Figure 1). Conversions of added substrates using this microfiltered cell-free extract can be easily visualized by HPLC analysis. Conventional dialysis is incapable of achieving such efficiency, and gel filtration with a G-25 column is more time consuming and requires prior concentration by which point substantial losses in protein activities have taken place. Following filtration dialysis, the cell-free extract has been shown to be stable for 4−7 days at 4 °C. If dialysis with this method is not carried out, all enzymatic activities in the biosynthetic pathway are lost within 12−24 h.

**Elucidation of the Cofactor Requirements of the Pathway.** As cell-free extracts prepared by this method are essentially "metabolite-free", we utilized preparations from wild-type *A. parasiticus* SU-1 cells to demonstrate the cofactor requirements of the pathway. Conversion of norsolorinic acid (**1**) to aflatoxin $B_1$ (**13**) can only be observed if NADPH and SAM are supplied to the extract in addition to the substrate as shown in the photograph of the TLC plate under long-wavelength UV light depicted in Figure 1. Lanes 1 and 2 are control lanes which demonstrate the effectiveness of the membrane filtration step. Whereas many metabolites are present in the crude extract (lane 1), the diafiltered protein suspension is substantially improved, exhibiting a very low background of contaminating metabolites (residual NOR is at the top of lane 2). When NOR (**2**) was added to the extract together with NADPH, SAM, and FAD, efficient conversion to $AFB_1$ resumed (lane 3). Omission of either NADPH (lane 4) or SAM (lane 5) from the extract was clearly detrimental to the overall conversion and $AFB_1$ could no longer be observed. This overall transformation [NOR (**1**) → $AFB_1$ (**13**)] also appears to exhibit a flavin dependence (Figure 1, lane 6) as exemplified by the decrease in biosynthesis of aflatoxin when FAD was withdrawn from the reaction mixture. It is perhaps not surprising that dependence on this cofactor appears weak as FAD is typically tightly enzyme

(22) Bhatnagar, D.; Ullah, A. H.; Cleveland, T. E. *Prepr. Biochem.* **1988**, *18*, 321−349.
(23) Yabe, K.; Ando, Y.; Hashimoto, J.; Hamasaki, T. *Appl. Environ. Microbiol.* **1989**, *55*, 2172−2177.
(24) Yu, J.; Cary, J. W.; Bhatnagar, D.; Cleveland, T. E.; Keller, N. P.; Chu, F. S. *Appl. Environ. Microbiol.* **1993**, *59*, 3564−3571.
(25) Yu, J.; Chang, P.-K.; Payne, G. A.; Cary, J. W.; Bhatnagar, D.; Cleveland, T. E. *Gene* **1995**, *163*, 121−125.
(26) Trail, F.; Mahanti, N.; Linz, J. *Microbiology* **1995**, *141*, 755−765.
(27) Chatterjee, M.; Townsend, C. A. *J. Org. Chem.* **1994**, *59*, 4424−4429.
(28) Watanabe, C. M. H.; Townsend, C. A. *J. Org. Chem.* **1996**, *61*, 1990−1993.
(29) Prieto, R.; Woloshuk, C. P. *Appl. Environ. Microbiol.* **1997**, *63*, 1661−1666.
(30) Yabe, K.; Ando, Y.; Hamasaki, T. *Appl. Environ. Microbiol.* **1988**, *54*, 2101−2106.
(31) Wan, N. C.; Hsieh, D. P. H. *Appl. Environ. Microbiol.* **1980**, *39*, 109−112.
(32) Anderson, M. S.; Dutton, M. F. *Appl. Environ. Microbiol.* **1980**, *40*, 706−709.
(33) Dutton, M. F.; Anderson, M. S. *Appl. Environ. Microbiol.* **1982**, *43*, 548−551.
(34) Scopes, R. K. *Protein Purification, Principles and Practice*; Springer-Verlag: New York, 1994; pp 317−324.

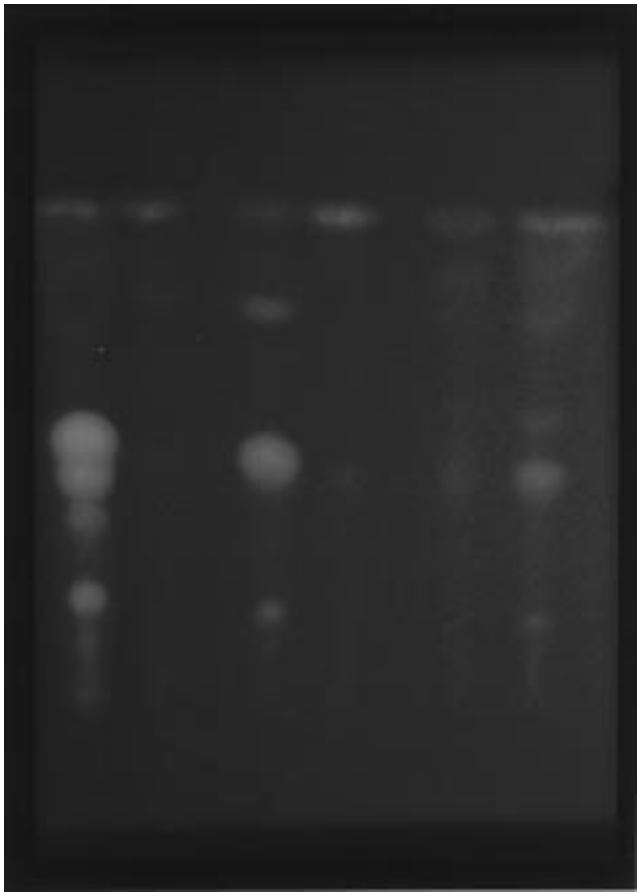


**Figure 1.** Metabolites extractable into ethyl acetate from (left to right) lane 1, crude cell-free extract (CFE); lane 2, CFE after diafiltration; lane 3, diafiltered CFE incubated ~8 h with added NOR (**2**), NADPH, SAM, and FAD; lane 4, diafiltered CFE ~8 h with added NOR (**2**), SAM, and FAD; lane 5, diafiltered CFE incubated ~8 h with added NOR (**2**), NADPH, and FAD; lane 6, diafiltered CFE incubated ~8 h with added NOR (**2**), NADPH, and SAM.

bound. In keeping with these results, analysis of the deduced polypeptide sequences of the *A. parasiticus* aflatoxin cluster genes[26] or *A. nidulans* sterigmatocystin cluster homologues[35] reveal several P-450 enzymes, an *O*-methyltransferase with a conserved *S*-adenosylmethionine binding site, and a single FAD monooxygenase that includes both an NADPH and FAD binding domain.

We investigated the cofactor requirements of three of the more difficult transformations of the pathway corresponding to the rearrangement of AVR (**4**) → VA (**9**), VA (**9**) → OMST (**12**), and OMST (**12**) → $AFB_1$ (**13**) with the hope of identifying postulated intermediates and to establish the functions of previously unassigned genes. All three transformations demonstrated the requirement for NADPH. When averufin (**4**) was supplied to a cell-free extract of SU-1 (wild-type *A. parasiticus* $AFB_1$ producer), in addition to added cofactors including NADPH, SAM, and FAD, the product mixture was found to contain aflatoxin (**13**) as well as trace quantities of metabolites such as VA (**9**) and ST (**11**). If NADPH was omitted from the reaction mixture, however, neither conversion of AVR (**4**) to VA (**9**) or to any other aflatoxin intermediate was observed, nor were any "new" products detected by HPLC analysis. Omission of FAD from the enzyme preparations clearly resulted in a substantial decrease in aflatoxin production but assignment of this cofactor to any one particular step of the pathway could not be made. Intermediates, while detectable, did not accumulate to any appreciable extent. Interestingly, if *S*-adenosylmethionine was omitted from the reaction mixture while supplying averufin (**4**) together with NADPH and FAD, a product formed with a xanthone-like chromophore [$\lambda_{max}$ = 248, 330 nm (±1 nm)] highly reminiscent of, but red-shifted from, OMST [**12**; $\lambda_{max}$ = 236, 309 nm (±1 nm)]. Isolation and $^1$H NMR spectral analysis of the compound revealed the absence of methoxyl resonances, and H-5 and H-8 had moved upfield. These observations and its molecular mass were entirely consistent with its assignment as DMST (**10**), in accord with expectation.[36]

In the final stage of biosynthesis, conversion of OMST (**12**) to $AFB_1$ (**13**), NADPH is the single cofactor required for this transformation.[37] If this cofactor was eliminated from the reaction mixture, *O*-methylsterigmatocystin (**12**) remained unaffected and turnover to aflatoxin (**13**) was not observed. Detection of the postulated hydroxylated intermediate, 10-hydroxy-*O*-methylsterigmatocystin, the proposed intermediate formed by oxidation by a P-450 monooxygenase) and "NIH-shift" of OMST (**12**), could not be made.[27,28] As it appears that a single enzyme is likely involved in this step of the pathway,[29] it is possible this intermediate remains substantially enzyme bound and is simply not detectably released in the course of two oxidative steps to $AFB_1$.[6]

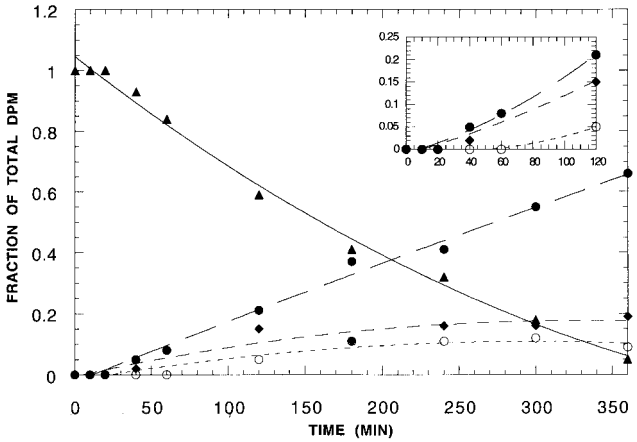



**Figure 2.** Time course of diafiltered CFE conversion of NOR (**2**) to $AFB_1$ (**13**): (▲) NOR; (●) VA; (◆), ST; (○) $AFB_1$.

**Time-Course of the Norsolorinic Acid to Aflatoxin $B_1$ Conversion.** To monitor the dynamics of the pathway as catalyzed by the cell-free system, we conducted a time-course experiment with the aim to observe the overall rise and fall of intermediates as a function of time. Radiolabeled NOR (**2**) was prepared by the administration of [1-$^{14}$C]acetate to the *A. parasiticus* norsolorinic acid-accumulating mutant NOR-1, and the radiolabeled **2** was isolated, purified, and recrystallized to constant specific activity. The labeled NOR (**2**) was then added to the cell-free extract together with all the necessary cofactors (NADPH, SAM, and FAD), and allowed to incubate for the desired length of time. A graphical representation of the results is shown in Figure 2. Norsolorinic acid (**2**) was steadily consumed over the course of the experiment (95% over 6 h). Intermediates were not detectable for 20−40 min from the point

(35) Brown, D. W.; Yu, J.-H.; Kelkar, H. S.; Fernandes, M.; Nesbitt, T. C.; Keller, N. P.; Adams, T. H.; Leonard, T. J. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 1418−1422.

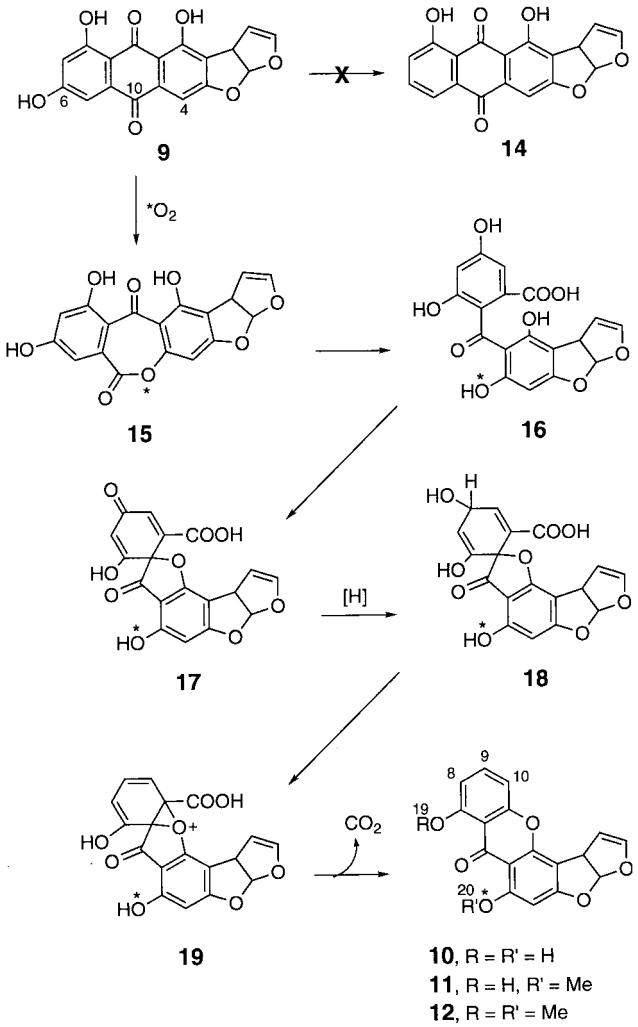
(36) Elsworthy, G. C.; Holker, J. S. E.; McKeown, J. B.; Robinson, J. B.; Mulheirn, L. J. *J. Chem. Soc., Chem. Commun.* **1970**, 1069−1070. See also: Cole, R. J.; Cox, R. H. *Handbood or Toxic Fungal Metabolites*; Academic Press: New York, 1981; pp 81 and 91.

(37) Cleveland, T. E.; Bhatnagar, D. *Can. J. Microbiol.* **1987**, *33*, 1108−1112.

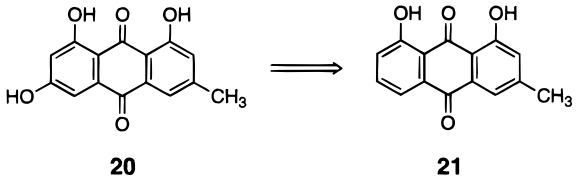
of addition, which may reflect the relatively poor solubility of the added NOR (**2**) in aqueous solution. Surprisingly few intermediates were observed at any time point, VA (**9**) and ST (**11**) being the only two metabolites that accumulated to any significant extent. No other intermediates, known or previously unknown, were convincingly detectable. As radiolabeled substrate was utilized in this experiment, the failure to see intermediates cannot be attributed to a lack of sensitivity of the method, but may reflect the relative kinetic ease or difficulty of the chemistry at hand. It is interesting that the intermediates that were observed to accumulate [VA (**9**) and ST (**11**)] occur late in the pathway where complex rearrangement processes take place and substantial changes must be made to the nuclear framework of these substrates. Up until that point, the intermediates [specifically those between NOR (**2**) and VA (**9**)] appear to be processed quite efficiently by the enzymes of the cell-free system such that detection of, for example, AVN (**3**), AVR (**4**), HVN (**5**) or VOAc (**6**) could not be made. While production of both ST (**11**) and $AFB_1$ (**13**) appeared to level off by 6 h of incubation, VA (**9**) was still clearly being produced in large amounts. Nonetheless, while the results of the time-course experiment were useful from the perspective of visualizing the overall dynamics of the pathway, we were surprised and disappointed by the failure to detect other known or proposed intermediates. Ironically the success of the new cell-free method apparently gives the majority of the biosynthetic enzymes in sufficient stability and amount to carry on their catalytic tasks efficiently without the detectable buildup of intermediates—apart from two chemically demanding nuclear rearrangement steps. Identification of pathway intermediates, which may be enzyme bound, and the mechanisms of the reactions that interrelate these will be deciphered by other means as the synthesis and testing of labeled potential precursors, and by the direct study of the enzymes involved which will in turn be available from classical biochemical techniques or protein expression methods.

**Examination of the Rearrangement of Versicolorin A to Demethylsterigmatocystin.** The oxidative rearrangement of versicolorin A (**9**) to demethylsterigmatocystin (**10**) is more complex than it at first seems (Scheme 2). Observed in the overall transformation is the loss of the C-6 hydroxyl of the tetrahydroxyanthraquinone of versicolorin A (**9**), an oxidative cleavage process resulting ultimately in the introduction of molecular oxygen at C-5 of DMST (**10**), as well as loss of a C-1 unit. Previously, aromatic deoxygenation had been thought to occur as a first step to give 6-deoxyversicolorin A (**14**), much as the ergochrome polyketide-derived fungal metabolite emodin (**20**) was successfully converted to chrysophanol (**28**) in cell-free extracts by an NADPH-dependent reductase (Scheme 3).[38] The failure of **14**, however, to show incorporation into aflatoxin undermined the likelihood of this mechanistic hypothesis, and it was concluded that deoxygenation must occur at a later stage in the biosynthesis.[39] To initiate the transformation then, the central ring of versicolorin A (**9**) could be envisioned to cleave by way of Baeyer−Villiger product **15** leading to the *o*-carboxybenzophenone intermediate **16** after hydrolysis. An $^{18}O$-labeling experiment has, in fact, established molecular oxygen (*O) at the methoxyl position, C-9, of aflatoxin (**13**) in support of such a mechanism.[28,40] Intramolecular phenolic coupling can be invoked to give the spirohexadienone **17** followed by

**Scheme 2**




**Scheme 3**




reduction, presumably in an NADPH-dependent step, to then allow both expulsion of the C-6 hydroxyl group (of **9**) and additional rearrangement, including loss of carbon dioxide, to produce demethylsterigmatocystin (**10**, Scheme 2). Although intermediacy of the spirohexadienone **17** has yet to be established, such an intermediate is presumed to be thermodynamically stable by analogy to the grieseofulvins.[41]

From a strictly genetic standpoint, two genes have been implicated in xanthone formation by gene disruption and gene complementation experiments encoding a P-450 monooxygenase[42] and a ketoreductase,[43] proteins responsible for the biochemical conversion of versicolorin A (**9**) to demethylsterigmatocystin (**10**). The putative P-450 monooxygenase could facilitate the initial Baeyer−Villiger oxidation step (to give **15**).

(38) Anderson, J. A.; Lin, B.-K.; Williams, H. J.; Scott, A. I. *J. Am. Chem. Soc.* **1988**, *110*, 1623−1624.

(39) Graybill, T. L.; Pal, K.; McGuire, S. M.; Brobst, S. W.; Townsend, C. A. *J. Am. Chem. Soc.* **1989**, *111*, 8306−8308.

(40) Nakashima, T. T.; Vederas, J. C. *J. Chem. Soc., Chem. Commun.* **1982**, 206−208.

(41) Turner, W. B.; Aldridge, D. C. *Fungal Metabolites II*; Academic Press: London, 1983; pp 121−123.

(42) Keller, N. P.; Segner, S.; Bhatnagar, D.; Adams, T. H. *Appl. Environ. Microbiol.* **1995**, *61*, 3628−3632.

(43) Skory, C. D.; Chang, P. K.; Cary, J.; Linz, J. E. *Appl. Environ. Microbiol.* **1992**, *58*, 3527−3537.

The ketoreductase, which contains a conserved NADPH binding domain, could then conceivably serve to catalyze the reduction of the spirohexadienone (**17**) to potentiate elimination of the C-6 hydroxyl of versicolorin A (**9**). Nonetheless considerable ambiguity resides in this step of the pathway and it is unclear what the precise functions are of the proteins encoded by these genes or whether additional enzymes are required. Overexpression of these biosynthetic genes or purification of the native enzymes for direct investigation may help to resolve this issue.

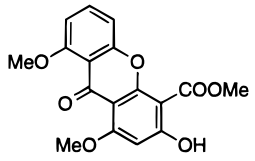
A deuterium-labeling experiment was performed to track the incorporation of isotope into *O*-methylsterigmatocystin (**12**) that was biosynthetically derived from versicolorin A (**9**, natural abundance) in $d_7$-glucose/$D_2O$ supplemented medium. The intent was to identify all positions arising from reductive processes and/or susceptible to solvent exchange. DIS-2, an *A. parasiticus* fatty acid synthase disruption mutant (*fas-1A*$^-$) that does not produce aflatoxin was utilized.[44] The fungus was initially cultured on Adye and Mateles medium[45] and subsequently transferred into a replacement medium made up in $D_2O$ and supplemented with $d_7$-glucose as the only carbon source at 36 h of growth. Following transfer, the mycelia were incubated for an additional 12 h at 30 °C to ensure proper equilibration. At 48 h, versicolorin A (**9**, 15 mg/250 mL flask) was solubilized in dimethylformamide and administered to the mycelial cultures. The cell suspensions were grown for an additional 12 h to obtain optimal production of *O*-methylsterigmatocystin (**12**). Extraction and purification of *O*-methylsterigmatocystin (**12**) from the DIS-2 mycelia and medium afforded ~4 mg of metabolite. The sample was analyzed by $^1$H NMR spectroscopy employing a relaxation time of 5 times the longest $T_1$ (5 s) to measure the extent of isotopic enrichment at each site with good accuracy (±3%).

**Table 1.** Sites of Deuterium Enrichment Observed in *O*-Methylsterigmatocystin

| compound[a] | position | % incorporation |
|---|---|---|
| *O*-methylsterigmatocystin | C-10 | 45 ± 3 |
| | C-9 | 64 ± 3 |
| | C-8 | 18 ± 3 |
| | C-19, C-20 | 51 ± 3 |

[a] *O*-Methylsterigmatocystin was isolated from DIS-2 cells supplemented with versicolorin A and cultured on a $d_7$-glucose/$D_2O$ medium.

The $^1$H NMR spectrum of deuterium-labeled *O*-methylsterigmatocystin (**12**) revealed five sites of deuterium incorporation, characterized by diminished proton signal intensity, corresponding to three of the aryl-ring carbon centers C-8, C-9, and C-10 and both methoxyl carbons, C-18 and C-19 (Table 1). Care was taken to ensure accurate $^1$H NMR assignments of H-8 and H-10. Through NOE measurements and comparisons to the model xanthone **22**,[46] these were found to agree with spectral designations made earlier by Steyn.[47] The results indicated a substantial incorporation of deuterium at positions C-10 (45 ± 3%) and C-9 (64 ± 3%) but moderate at C-8 (18 ± 3%). The deuterium label at both methoxyl positions of *O*-methylsterigmatocystin (**12**), C-19 and C-20 (51 ± 3%), must derive from isotopically labeled *S*-adenosylmethionine. The high extent of labeling in ring A of OMST (**12**) was wholly unexpected,




**22**

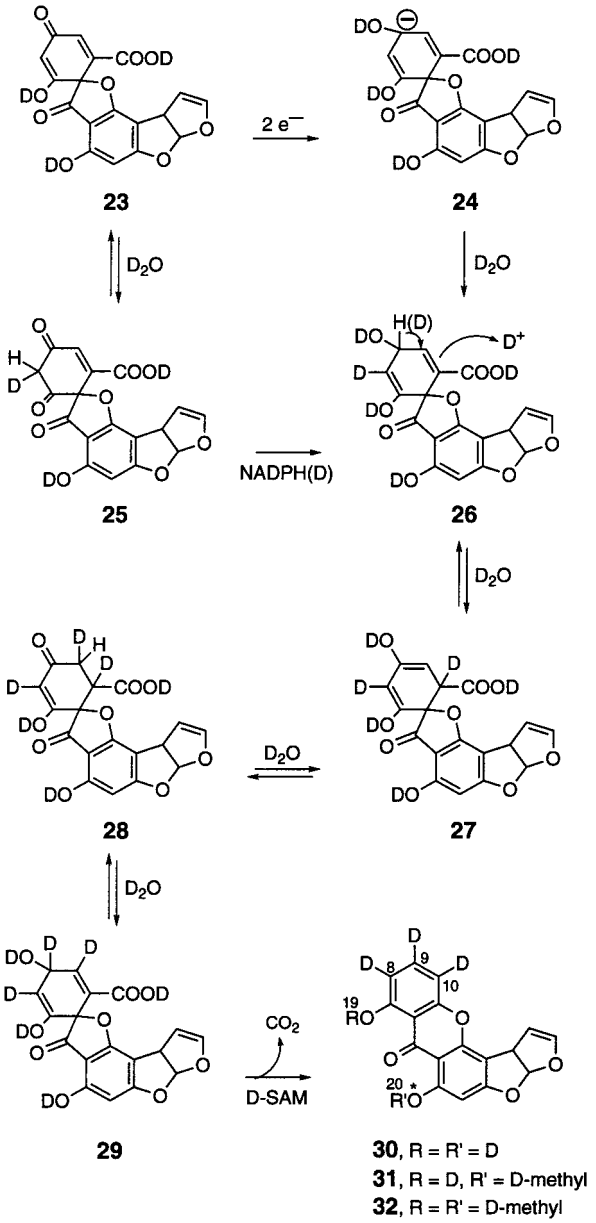
particularly at C-9, which corresponds to the hydroxylated carbon C-6 in versicolorin A (**9**).

The appearance of deuterium at C-9 of OMST (**12**) would be thought to arise from NADPD synthesized in the deuterium-rich environment of the cell. In an attempt to differentiate between deuteriation from reduction with NADPD and from exchange with the $D_2O$ medium, the suspended-cell experiment above was repeated in the presence of $d_7$-glucose and normal water. $^1$H NMR spectral analysis of the isolated **12** revealed no detectable deuterium incorporation at any of the five sites labeled previously. Differences in integrated intensities from $^1$H NMR spectroscopy inherently lack sensitivity, however, to detect low levels of deuterium. The sample was examined by mass spectrometry, therefore, and compared to an identical sample at natural abundance. The isotopic cluster following the parent ion showed 5.5% $d_1$, 3% $d_2$, and <1% $d_3$ species were present (±1%, average of 10 scans). The M-15 peak, presumably derived from loss of methyl from one of the methoxyl groups, gave the following intensities: 5% $d_1$, 2% $d_2$, and <1% $d_3$. If it is assumed from the $d_1$ contents of the M and M-15 species that each methoxyl is labeled with deuterium at approximately 0.5%, the maximum label that could possibly be present at C-9 is <5%—far less than the 64% observed in a $D_2O$-containing medium.

The results of the two whole-cell experiments above suggest that deuterium incorporation at C-9 in ring A of OMST (**12**) arises through exchange with the medium—an event that must occur *subsequent* to reduction at this center. Such a process is not easily rationalized. To establish directly the role of NADPD in the conversion of VA (**9**) to OMST (**12**), the new cell-free method was brought to bear. The membrane microfiltration step removes small molecules, and, therefore, successful conversion of **9** to **12** would depend entirely on added reductant. Both A-side- and B-side-labeled NADPD were prepared[48,49] and supplied to large-scale cell-free extracts in addition to versicolorin A (**9**, natural abundance), unlabeled *S*-adenosylmethionine and all cofactors required to carry out the transformation. The reactions were carried out at 30 °C for 36 h prior to product extraction and purification. Approximately 500 μg of *O*-methylsterigmatocystin (**12**) was obtained in each case, and the isolated metabolites were analyzed by $^1$H NMR spectroscopy. Neither *O*-methylsterigmatocystin sample (prepared from A-side or B-side NADPD) convincingly showed deuterium enrichment anywhere in the molecule in agreement with the suspended-cell experiments described above. The samples were, therefore, submitted for mass spectral analysis. Isotopic abundance calculations (average of 10 scans) were performed and clearly showed that both *O*-methylsterigmatocystin samples were singly labeled with deuterium, exhibiting isotopic enrichments of 7 ± 1% (B-side preparation) and 2 ± 1% (A-side preparation), respectively. Neither doubly nor triply labeled species were detected. To ensure the reproducibility of these results, the entire experiment was repeated and again showed a 7 ± 1%

(44) Mahanti, N.; Bhatnagar, D.; Cary, J. W.; Joubran, J.; Linz, J. E. *Appl. Eviron. Microbiol.* **1996**, *62*, 191−195.
(45) Adye, J.; Mateles, R. I. *Biochim. Biophys. Acta* **1964**, *86*, 418−420.
(46) Casillas, L. N.; Townsend, C. A. Unpublished results.
(47) Pachler, K. G. R.; Steyn, P. S.; Vleggaar, R.; Wessels, P. L.; Scott, D. B. *J. Chem. Soc., Perkin Trans. 1* **1976**, 1182−1189.
(48) Viola, R. E.; Cook, P. F.; Cleland, W. W. *Anal. Biochem.* **1979**, *96*, 334−340.
(49) *Sigma Quality Control Test Procedure for enzymatic assay of alcohol dehydrogenase, NADP*$^+$ *dependent*; Sigma Chemical Co.: St. Louis, MO.

**Scheme 4**




and 2 ± 1% incorporation for the B-side and A-side preparations of NADPD in *O*-methylsterigmatocystin (**12**). As enzymatic reactions are generally observed to be stereospecific processes, the 2% enrichment observed with the A-side-labeled cofactor may have arisen from stereochemically impure reductant or conceivably the transfer of deuterium to the B face of NADP generated in the extract over the course of reaction (36 h incubation period). An attempt was made to assign the position of label to the A ring of *O*-methylsterigmatocystin (**12**) on the basis of its mass fragmentation pattern but the background was too high for this to be accomplished.

We have unambiguously demonstrated that NADPH is a necessary cofactor to achieve the cell-free rearrangement of VA (**9**) to OMST (**12**), and yet when this cofactor (the sole reductant) was provided in deuterated form, it labeled the product only to very small extent. The majority of hydrogen at C-9 in **12** arises from the medium. It is possible to imagine this process as a two-electron reduction of the spirodienone **23** to give the doubly allylic anion **24**, which upon protonation/deuteriation would afford **26** (Scheme 4). But NADPH is typically associated with discrete hydride transfer. Moreover, some residual deuterium derived from stereospecifically labeled NADPD resides at a specific site in OMST (**12**), presumably C-9, as evidenced in the cell-free experiments. An alternative mechanism would require extensive exchange with the medium at C-9 *after* reduction has occurred at this site, but *before* aromatization takes place to halt such a process. We advance (with due skepticism) a tentative scheme to account for all of these observations (Scheme 4). Spirodieneone **23** can easily exchange with the medium at what will become C-8 in the product through simple enolization shown in **25**. It is proposed next that reduction by NADPH(D) occurs to afford the spirodienol **26**. Degenerate allylic rearrangement between **26** and **27** would give exchange at the carbon destined to become C-9 in OMST. Similarly, enolization of **27** (to **28**) would give rise to exchange at C-10. C-9 and C-10 are the principal sites of apparent exchange with the medium. Finally, migration of the spiroaryl ether, loss of water, and decarboxylation can be invoked to yield OMST (**12**).

## Conclusion

The extensive proton exchange in the A ring during the skeletal rearrangement process of VA (**9**) to DMST (**10**), particularly at the site of formal reduction, C-9, is surprising. Genetic evidence points to at least two proteins being involved in the conversion.[42] More detailed understanding of this process will come from examination of the chemical fate of specific intermediates in the overall transformation and direct study of the proteins involved. These unexpected findings not withstanding, the procedural ease of the new cell-free extract preparation and the analytical advantage of essentially complete removal of small molecules ensures the testing of potential intermediates, cofactors, and medium effects in the absence of background reactions of endogenous materials. Moreover, the biosynthetic proteins themselves have been shown to be dramatically stabilized by this procedure and have made possible the observation of the entire biosynthetic pathway to aflatoxin for the first time in a cell-free system. With ∼15 chemical steps involved, some quite complex, we believe this is a significant methodological advance that will benefit not only the investigation of aflatoxin biosynthesis, but will prove generally applicable to a wide range of biological systems.

## Experimental Section

**Instrumentation and General Methods.** $^1$H NMR spectra were recorded on a Varian XL-400 specrometer operating at 400 MHz. Chemical shifts are reported in ppm referenced to chloroform (7.26 ppm). The filtration unit (Amicon RA 2000) utilized for clarification and dialysis of cell-free extracts was obtained from Millipore (Bedford, MA). Silica gel TLC plates (250 $\mu$m) were purchased from Analtech (Newark, DE). Flash chromatography was performed using 200−240 mesh Merck silica gel. Preparative HPLC separations were achieved with a semiprep Phenomenex Partisil 10 ODS-3 C-18 column (250 × 9.40 mm) on a Varian 5020 liquid chromatograph with an attached ABI (model 1000S) diode array detector. Analytical separations, utilized in enzyme assay procedures, were performed with a Phenomenex Spherex C-18 column (250 × 4.60 mm). [1-$^{14}$C]Acetic acid, sodium salt was obtained from Moravek Biochemicals (Brea, CA). $D_2O$ (99.9 atom %) was purchased from Isotec, Inc. (Miamisburg, OH). $d_7$-Glucose (98 atom %) was obtained from Cambridge Isotopes (Andover, MA). $d_6$-Ethanol (99 atom %) was obtained from Stohler Isotope Chemicals (Waltham, MA). $d_8$-2-Propanol (99+ atom %) and all enzymes utilized in the preparation of A-side and B-side NADPD were purchased from Sigma (St. Louis, MO). AG MP-1, the strongly basic macroporous resin used in the purification of the nucleotides, was available from Bio-Rad (Hercules, CA).

**Organisms.** The *A. parasiticus* wild-type strain SU-1 (ATCC 56775), the versicolorin A-accumulating mutant WH-1 (ATCC 36537),

the norsolorinic acid generating strain NOR-1 (ATCC 24690), and the *O*-methylsterigmatocystin-producing mutant SRRC 2043 (ATCC 62882) were obtained from the American Type Culture Collection (ATCC; Rockville, MD). The *A. parasiticus* fatty acid synthase disruption mutant strain DIS-2 was a generous gift from Professor J. E. Linz (Michigan State University).

**Media.** The Adye and Mateles (AM) growth medium[45] used to culture the mycelial strains described above contained per liter sucrose, 50 g; potassium phosphate monobasic, 10 g; ammonium sulfate, 3 g; magnesium sulfate (anhydrous) 1 g; and trace metals, 2 mL. The replacement medium (RM)[50] contained per liter ($D_2O$) glucose or $d_7$-glucose, 1.62 g; potassium phosphate monobasic, 5 g; potassium chloride, 0.5 g, magnesium sulfate (anhydrous), 0.25 g; and trace metals, 2 mL.

The AM growth medium (one liter, in 4-L Erlenmeyer flasks) was autoclaved (140 °C, 20 psi) for 20 min and cooled to room temperature prior to inoculation. The deuterated replacement medium was sterilized by filtration through a 0.22 $\mu$m filtration unit (Corning; Corning, NY) prior to use.

**Culture Techniques.** All fungi were grown on potato dextrose agar plates[50] for 4−7 days. The agar plates contained yeast extract, 2.5 g; Bacto-agar, 2.5 g; potato dextrose agar, 19 g (Difco; Detroit, MI); and distilled water, 500 mL (20 plates). The spores were suspended in a solution of Tween 80 (0.05% v/v), 9 mL, which was subsequently diluted in NaCl (0.85% w/v), 9 mL. A fraction of the spore suspension (3 mL) was then transferred to 1 L of AM medium in 4-L Erlenmeyer flasks unless stated otherwise. The flasks were incubated at 28−30 °C in the dark at 175 rpm for the requisite amount of time.

**Cell-free Extract Preparation. 1. *NOR* → $AFB_1$ Transformation.** *A. parasiticus* SU-1 cells were cultured for 84 h on AM growth medium as described above. The mycelia were then vacuum filtered in a Buchner funnel layered (4×) with cheesecloth and rinsed thoroughly with distilled water. For each cell-free extract, 50 g of cells (damp weight) were flash frozen in liquid nitrogen and pulverized in an Osterizer blender with dry ice (cells were ground for 2 min in 30 s cycles). The powdered mycelia were then suspended in 300 mL of buffer [50 mM potassium phosphate, pH 7.5; 30% glycerol; 2 mM $\beta$-mercaptoethanol; 100 $\mu$M phenylmethylsulfonyl fluoride (predissolved in a minimal amount of acetone); 100 $\mu$M benzamidine and 1 mM EDTA]. The mixture was stirred at 4 °C for 2 h and the cell suspension was subsequently centrifuged for 20 min at 20 000*g*. The decanted supernatant served as the crude cell-free extract. The protein extract was then filtered through four layers of cheesecloth to remove any residual cell debris, followed by dialysis (against 4 L of buffer) with the Amicon RA 2000 apparatus employing a 10 000 molecular weight cutoff membrane to produce the metabolite-free cell-free extract in a volume of about 175 mL.

**2. VA → OMST Transformation.** *A. parasiticus* SRRC 2043 cells (84 h) were used to prepare cell-free extracts utilized in NADPD-labeling experiments. The extract was prepared as described above.

**CFE Enzyme Assays. *NOR* → $AFB_1$ Transformation.** To the metabolite-free SU-1 cell-free extract (5 mL) was added 20 $\mu$L of a 1 mg/mL solution (in acetone) of NOR (**2**), or any other aflatoxin biosynthetic intermediate of interest and 40 $\mu$L of cofactor solution (1 mg NADPH; 1 mg SAM and 1 mg FAD in 1 mL of distilled water). Reactions were typically incubated with mild shaking at 30 °C for ∼8 h to ensure maximal turnover of NOR (**2**) to $AFB_1$ (**13**). Reactions were quenched by vortexing with 1 mL of ethyl acetate. Emulsions were eliminated by microcentrifugation of the samples at 14 000 rpm for 2 min. The organic layer was then transferred to a fresh Eppindorf tube and evaporated to dryness. Samples were solubilized with 20 $\mu$L of ethyl acetate and subjected to TLC analysis or dissolved in 50 $\mu$L of acetonitrile and analyzed by HPLC.

**TLC Analyses.** For chromatographic identification of OMST (**12**) and $AFB_1$ (**13**) a ternary mixture of 6:3:1 chloroform/ethyl acetate/formic acid was utilized. All other intermediates were visualized with a solvent mixture of 8:1.8:0.2 hexane/ethyl acetate/acetic acid. Aflatoxin and its intermediates can be easily visualized under longwave UV light.

(50) Brobst, S. W.; Townsend, C. A. *Can. J. Chem.* **1994**, *72*, 200−207.

**3. HPLC Analyses.** Separation of aflatoxin and its intermediates was achieved with a gradient that extended from 75% A/25% B → 40% A/60% B (A, 0.1% trifluoroacetic acid/water; B, acetonitrile) in 55 min. Samples were run at constant flow rate of 1 mL/min and monitored at $\lambda$ 310 nm.

**NOR (2) → $AFB_1$ (13). 1. Time-Course Analysis.** SU-1 cell-free extract devoid of small molecules was prepared as described above. To 175 mL of extract was added 1.05 mL of $^{14}$C-labeled norsolorinic acid (1 mg/mL, in acetone) and 1.4 mL of cofactor solution which contained 1 mg of NADPH, 1 mg of SAM, and 1 mg of FAD in 1 mL of distilled water. Aliquots (5 mL) were removed and quenched at specific time points (see Figure 2) by vortexing with 1 mL of ethyl acetate. The samples were microcentrifuged, and the organics were transferred to fresh Eppindorf tubes and subsequently evaporated to dryness. Reactions were resolubilized with 50 $\mu$L of acetonitrile and analyzed by HPLC.

**2. HPLC Analyses.** To achieve separation of aflatoxin and its intermediates, a linear gradient was employed that extended from 75% A/25% B → 40% A/60% B (A, 0.1% trifluoroacetic acid/water; B, acetonitrile) in 55 min. The solvent composition was then held at 40% A/60% B from 55 to 59 min followed by a mixture of 20% A/80% B which was initiated at 60 min from the point of injection. Samples were analyzed by monitoring at $\lambda$ 310 nm (flow rate 1 mL/min). These lengthy run times were necessary to achieve separation of the broad range of intermediates that vary in polarity and comprise the aflatoxin pathway.

**Biosynthesis of Radiolabeled Norsolorinic Acid.** *A. parasiticus* NOR-1 cells were initially cultured on AM medium by inoculating six 500-mL Erlenmeyer flasks each containing 125 mL of medium with 375 $\mu$L of the spore suspension. The mycelia were allowed to culture for 35 h at 30 °C, shaking at 175 rpm. At 35 h of growth the mycelial suspensions were vacuum filtered on cheesecloth, rinsed thoroughly with distilled water, and subsequently transferred into RM medium (500-mL flasks each containing 125 mL of medium) supplemented with 5 g of glucose/L. To each flask was then added 80 $\mu$Ci of acetic acid, sodium salt (1 mCi/1 mL of distilled water). The cells were allowed to grow for an additional 65 h at 30 °C, 175 rpm. A second addition of radiolabeled substrate (80 $\mu$Ci [1-$^{14}$C]acetate/flask) was supplied 5 h after the first 40 h of growth. The mycelial mats were harvested by vacuum filtration, rinsed with distilled water, flash frozen in liquid nitrogen, and steeped in acetone overnight. Metabolites were also extracted from the media with ethyl acetate. The organic extracts from the cells and the mediium were consolidated and concentrated in vacuo. Purification of the labeled NOR (**2**) was most easily achieved by direct recrystallization from acetone. The material was recrystallized to constant specific activity (0.18Ci/mol). **Norsolorinic acid** $^1$H NMR (400 MHz, $d_6$-DMSO, natural abundance) $\delta$: 7.12 (s, 1H, H-4), 7.05 (d, $J$ = 2.4 Hz, 1H, H-5), 6.53 (d, $J$ = 2.4 Hz, 1H, H-7), 2.73 (t, $J$ = 7.2 Hz, 2H, H-2′), 1.59 (m, 2H, H-3′), 1.25 (m, 4H, H-4′/5′), 0.81 (t, $J$ = 6.8 Hz, 3H, H-6′).

**Biosynthesis of Versicolorin A.** Versicolorin A (**9**) was prepared by culturing Wh-1 cells (ATCC 36537) in four 4-L Erlenmeyer flasks each containing 1 L of AM growth medium as described above. At 84 h of growth, the mycelia were vacuum filtered, rinsed with distilled water, and flash frozen in liquid nitrogen. The mycelia were then steeped in acetone overnight, the organic extracts were concentrated in vacuo, and lyophilized to dryness. Purification was achieved by flash silica column chromatography (8:1.8:0.2: hexane/ethyl acetate/acetic acid). **Versicolorin A** $^1$H NMR (400 MHz, $d_6$-DMSO) $\delta$: 12.29 (s, 1H, OH), 12.00 (s, 1H, OH), 11.12 (br s, 1H, OH), 7.18 (s, 1H, H-4), 7.14 (br d, $J$ = 2.5 Hz, 1H, H-5), 6.94 (d, $J$ = 7.1 Hz, 1H, H-1′), 6.72 (t, $J$ = 2.4 Hz, 1H, H-4′), 6.59 (br d, $J$ = 2.5 Hz, 1H, H-7), 5.41 (t, $J$ = 2.4 Hz, 1H, H-3′), 4.77 (dt, $J$ = 7.1, 2.4 Hz, 1H, H-2′).

**Cell-Free Preparation of Demethylsterigmatocystin (10).** The diafiltered SU-1 cell-free extract was prepared as described above. To 175 mL of cell-free extract were added 700 $\mu$L of versicolorin A (1 mg/mL in acetone) and 1.4 mL of cofactor solution which contained 1 mg of NADPH and 1 mg of FAD in 1 mL of distilled water. The rection was allowed to incubate at 30 °C for 24 h. The metabolites were extracted from the protein suspension with ethyl acetate and the organic extracts were concentrated in vacuo. Isolation of DMST (**10**)

was carried out by HPLC employing an isocratic gradient of 40% A/60% B (A, 0.1% trifluoroacetic acid/water; B, acetonitrile). Samples were analyzed by monitoring absorption at $\lambda$ 310 nm (flow rate 1 mL/min). **Demethylsterigmatocystin** $^1$H NMR (400 MHz, $d_6$-acetone) $\delta$: 7.74 (t, $J$ = 8.4 Hz, 1H, H-9), 7.08 (br d, $J$ = 8.4 Hz, 1H, H-10), 6.95 (dd, $J$ = 0.6, 7.1 Hz, 1H, H-1′); 6.82 (br d, $J$ = 8.4 Hz, 1H, H-8); 6.38 (s, 1H, H-4); 6.15 (dd, $J$ = 2.0, 2.8 Hz, 1H, H-4′); 5.56 (dd, $J$ = 2.4, 2.8 Hz, 1H, H-3′), 4.92 (dddd, $J$ = 0.6, 2.0, 2.4, 7.1 Hz, 1H, H-2′). Mass spectrum (*m*/*z*): 310.

**Biosynthesis of Deuterium-Labeled *O*-Methylsterigmatocystin from a $d_7$-Glucose/$D_2O$-Supplemented Medium.** Dis-2 cells were initially cultured in 4-L Erlenmeyer flasks each containing 1 L of AM growth medium as described above. At 36 h of growth, the mycelia were vacuum filtered, and the cells (5 g) were then transferred into eight 250-mL Erlenmeyer flasks each containing 62.5 mL of RM medium. Versicolorin A (15 mg) was dissolved in 1.5 mL of dimethylformamide and added to each 5 g cell suspension at 48 h of growth. The cell suspensions were cultured for an additional 12 h and vacuum filtered and washed. The mycelia were then flash frozen in liquid nitrogen and steeped in acetone overnight. The acetone extract was concentrated in vacuo, lyophilized, and combined with the chloroform extracts of the medium. Partial purification of *O*-methylsterigmatocystin was achieved by flash chromatography on silica gel utilizing a gradient of [8:1.8:0.2 hexane/ethyl acetate/acetic acid (results in removal of an orange band, excess versicolorin A) followed by 6:3:1 chloroform/ethyl acetate/formic acid]. HPLC was then carried out employing an isocratic gradient of 40% A/60% B (A, 0.1% trifluoroacetic acid/water; B: acetonitrile) to yield purified OMST. **O-Methylsterigmatocystin** $^1$H NMR (400 MHz, $d_6$-acetone) $\delta$: 7.57 (t, $J$ = 8.4 Hz, 1H, H-9); 7.02 (dd, $J$ = 8.4 Hz, 1.2 Hz, 1H, H-10); 6.88 (dd, $J$ = 0.6, 7.1 Hz, 1H, H-1′); 6.86 (d, $J$ = 8.8 Hz, 1H, H-8); 6.59 (dd, $J$ = 2.0, 2.8 Hz, 1H, H-4′); 6.49 (s, 1H, H-4); 5.50 (dd, $J$ = 2.4, 2.8 Hz, 1H, H-3′); 4.85 (dddd, $J$ = 0.6, 2.0, 2.4, 7.1 Hz, 1H, H-2′); 3.86 (s, 6H, H-15/16).

**Preparation of A-Side-Labeled NADPD.**[49] To a 50-mL round-bottomed flask was added $NADP^+$, 50 mg; $d_8$-2-propanol, 1.55 g; alcohol dehydrogenase from *Thermoanaerobium brockii*, 50 U; and 100 mM Tris-HCl (pH 8). The reaction was stirred at 40 °C for 10 min and subsequently quenched by stirring with 1 mL of methylene chloride. The progress of the reaction had been monitored by UV by monitoring the increase in aborbance at $\lambda$ 340 nm. The aqueous layer was then decanted and lyophilized to dryness. Purification was achieved with an AG-MP-1 column (1 × 30 cm), a strongly anionic macroporous resin. Following equilibration with 200 mM LiCl (pH 10), the A-side-labeled NADPD [solubilized in 5 mL of 200 mM Li Cl (pH 10)] was loaded onto the column by gravity. Residual unreacted $NADP^+$ was eluted with ~200 mL of 200 mM LiCl (pH 10). $NADP^+$ had been shown to travel with the solvent front. NADPD was then released from the column by applying a gradient which extended from 200 mM → 500 mM LiCl (pH 10, 250 mL of each salt concentration). Fractions were monitored by UV spectroscopy. Samples containing purified NADPD exhibited $A_{260}/A_{340} \leq 2.3$. While both $NADP^+$ and NADPD absorb at $\lambda$ 260 nm, only NADPD absorbs at $\lambda$ 340 nm. Hence, the ratio $A_{260}/A_{340}$ gives a direct measure of the extent of the reaction.

**Preparation of B-Side-Labeled NADPD.**[48] To a 50-mL round-bottomed flask was added $NADP^+$; 50 mg; $d_1$-glucose, 12 mg; dimethyl sulfoxide, 6.7 mL; 83 mM potassium phosphate buffer (pH 8), 10 mL; and glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*. Mixing of potassium phosphate buffer and DMSO is exothermic and should therefore be combined and allowed to cool to room temperature prior to the addition of the substrates and enzyme. The reaction was stirred at room temperature for 24 h and quenched with 1 mL of methylene choride. The aqueous layer was lyophilized, and the resulting solid purified on an AG MP-1 column as described above. Fractions containing purified NADPD exhibited $A_{260}/A_{340} < 2.3$.

**Biosynthesis of Deuterium-Labeled *O*-Methylsterigmatocystin, from A-Side- and B-Side-Labeled NADPD.** The experimental procedures utilized for the production of OMST from A-side- and B-side-labeled NADPD were identical. NADPD (15 mg/LiCl solution), either A-side or B-side labeled, SAM (5 mg), and FAD (10 mg) were added to cell-free extracts prepared from 50 g of SRRC 2043 cells, in addition to 1 mg of versicolorin A (in 1 mL of acetone). Two 50-g cell-free extracts were utilized for each experiment (A face and B face). The reactions were incubated at 30 °C for 36 h, mixed by shaking at 100 rpm, and subsequently quenched by extracting with ethyl acetate. The pooled organic extracts were dried over anhydrous magnesium sulfate and concentrated in vacuo. HPLC purification of the crude material resulted in ~500 $\mu$g of purified A-side- or B-side-labeled *O*-methylsterigmatocystin.

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