Enzymology and Molecular Biology of Aflatoxin Biosynthesis

Robert E. Minto* and Craig A. Townsend*

Department of Chemistry, The Johns Hopkins University, Baltimore, Maryland 21218

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Rob Minto was born and raised in Ontario, Canada. He obtained an Honours B.Sc. in Applied Chemistry (Co-op) from the University of Waterloo in 1989. Upon receiving an NSERC (Canada) 1969 Scholarship, he joined the laboratory of Professor R. G. Bergman at the University of California, Berkeley, where he focused on the physical organic chemistry of enediyne cyclizations and was awarded a Ph.D. in 1994. From 1994 to 1997, he was a postdoctoral fellow with Professor C. A. Townsend at The Johns Hopkins University where he studied aflatoxin biosynthesis. In 1997, he joined the Chemistry and Biochemistry faculty of Miami University, Oxford, OH, as an Assistant Professor. His research program examines unusual organic reaction mechanisms in plants and fungi. Outside the lab, his interests include rockclimbing, canoeing, and backpacking.

Craig Townsend was born in Chicago and educated at Williams College. He received his Ph.D. with Ian Scott at Yale and was an International Exchange Postdoctoral Fellow of the Swiss National Science Foundation in the laboratory of Duilio Arigoni at the Eidgenössische Technische Hochschule in Zürich. He was appointed to the Department of Chemistry at The Johns Hopkins University in 1976 and has held the rank of Professor since 1985. From 1990 to 1994 he served as Chairman of the Department and currently holds joint appointments in the Departments of Biology and Biophysics. His research interests are broadly in bioorganic and natural products chemistry, but tempered by his family, research group, outdoor exercise, and the visual arts.

I. Introduction

Identified in 1960 as the cause of the "Turkey X Disease", aflatoxin B_1 (AFB_1, **1**, Scheme 1) has become the paradigm mycotoxin.[1] It is produced by *Aspergillus parasiticus*, *Aspergillus flavus*, and *Aspergillus nomius*, molds of wide distribution that infect grains and nuts and lead to contamination of the food supply.[2−4] The mutagenic and carcinogenic effects of aflatoxin in humans and other vertebrates have been studied extensively and are understood to involve oxidative activation by cytochromes P450 in the liver and kidney to yield *exo*-epoxide **2**.[5,6] The absolute configuration of this lipophilic and largely planar species is diabolically matched to the helical twist of DNA to provide, through facile intercalation, a potent electrophile in the major groove that reacts with the N-7 of guanine residues yielding covalent adducts as **3**.[7−11] A direct relationship between its ability to cause DNA damage and the incidence of human cancers has been identified in a mutational "hot spot" in the p53 gene observed to be unusually reactive with epoxide **2**.[12−14] Transversions G249T or C that occur during faulty repair at this locus give rise to the same amino acid change in the translated protein (Arg→Ser), which is incompetent in its regulatory role. Mutations or alteration in the expression

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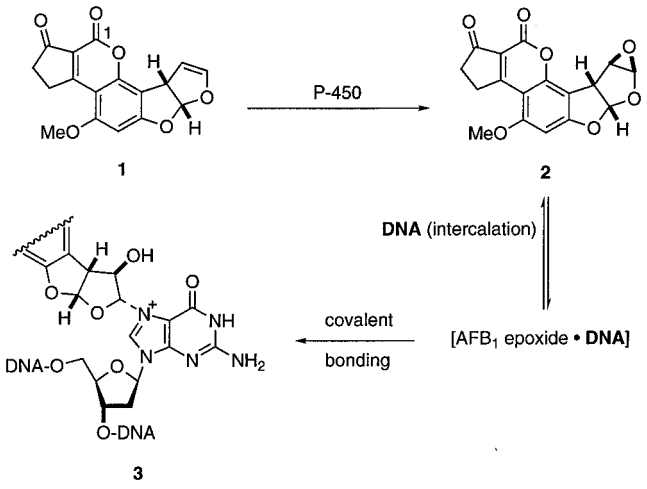
Scheme 1



level of p53 are associated with 40−50% of human cancers.[15−17]

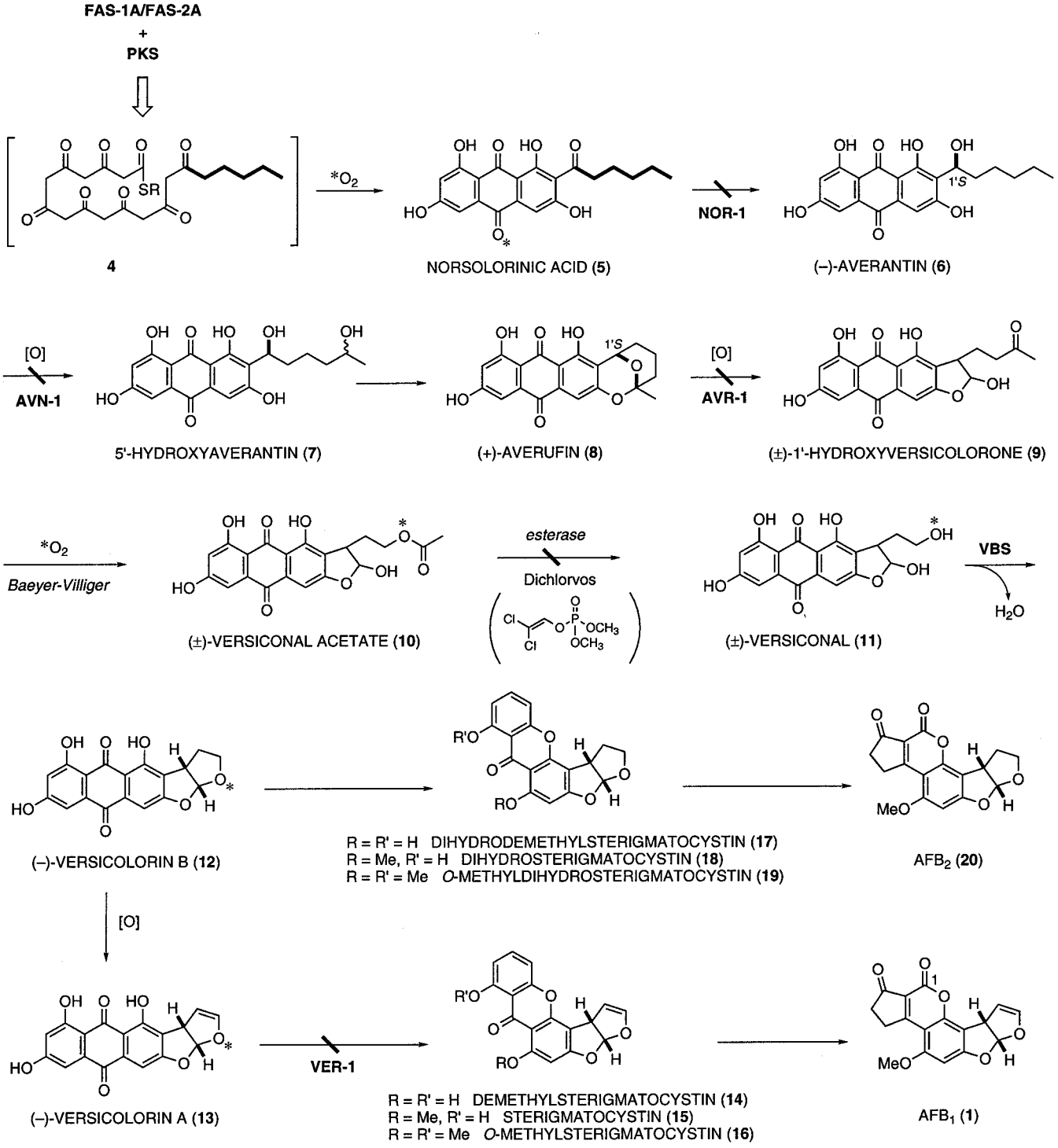
Among polyketide-derived natural products the biosynthesis of the aflatoxins is long and complex, and characterized by diverse oxidative rearrangement processes. Many of these appear representative of similar interconversions evident in the formation of other acetogenins. The current state of knowledge of the aflatoxin pathway is set out in Scheme 2. Landmark experiments by Büchi established the polyacetate origins of AFB_1 by classical means.[18,19] Subsequently, mutants of *A. parasiticus* blocked in their ability to form AFB_1 were generated that accumulated norsolorinic acid (NA, **5**),[20] averantin (AVN, **6**),[21] averufin (AVR, **8**),[22] 1′-hydroxyversicolorone (HVN, **9**),[23] and versicolorin A (VA, **13**).[24] Exposure of wild-type *A. parasiticus* to parts per million concentrations of the phosphorus-based insecticide Dichlorvos leads to the greatly reduced production of aflatoxin and the accumulation of the orange anthraquinone pigment versiconal acetate (**10**).[25,26] The xanthone sterigmatocystin (ST, **15**) is a known metabolite of the allied *Aspergillus* strains *versicolor* and *nidulans*. Using the sterigmatocystin producers, Dichlorvos and some of the blocked mutants above, radiolabeled acetate was incorporated into compounds **5**, **6**, **8**, **10**, **13**, and **15**.[21, 27−31] Uptake of these ^{14}C-labeled anthraquinones by wild-type *A. parasiticus* gave positive incorporations of radioisotope into AFB_1. Although specificity of labeling could not be established in these experiments, the increasing efficiency of incorporation from **5** to **15** into AFB_1 relative to acetate itself was supportive of their roles as biosynthetic intermediates.

A second line of research carried out principally by Steyn's group in South Africa established a common polyketide folding pattern in many of the intermediates shown in Scheme 2. By identifying the $^{13}C-^{13}C$ coupling partners from [1,2-$^{13}C_2$]acetate in **5**, **6**, **8**, **10**, **13**, **15**, and **1**, a self-consistent pattern of acetate incorporation was defined through these anthraquinone, xanthone, and coumarin structures in agreement with a hypothetical progenitor polyketide folded as shown in **4**.[32−37] Subsequent experiments with specifically ^{13}C-, $^{13}C-^{13}C$-, $^{2}H-^{13}C$-, $^{18}O-^{13}C$-labeled samples of averufin (**8**),[38−42] 1′-hydroxyversicolorone (**9**),[43] versiconal acetate (**10**),[44−46] versiconal (**11**),[44,46,47] versicolorin B (**12**),[44] and versicolorin A (**13**)[44] unambiguously established: (a) the intermediacy of these anthraquinone metabolites in the biosynthesis of AFB_1, (b) defined the oxidative steps in formation of the unique dihydrobisfuran ring system present in AFB_1, and (c) established the key metabolic branching event in the oxidative desaturation of versicolorin B (**12**) to versicolorin A (**13**) to give rise to the tetrahydrobisfuran (relatively noncarcinogenic) and dihydrobisfuran (acutely carcinogenic) series of late metabolites **12**→**20** and **13**→**1**, respectively.

An important early insight into the first steps of precursor assembly in aflatoxin biosynthesis came with the unexpectedly efficient, intact incorporation of hexanoic acid.[48,49] This observation was followed by attempted incorporations of acetate, butanoate, hexanoate, and 3-oxooctanoate (Scheme 3) as their *N*-acetylcysteamine thioesters (NAC).[50] While butanoate gave only β-oxidation and secondary incorporation as acetate, hexanoylNAC gave an impressive 22% specific incorporation into averufin (hence aflatoxin) compared to a 3−4% intact incorporation by hexanoate under identical conditions. Experiments with [1-^{13}C,$^{18}O_2$]hexanoate eliminated degradation of higher fatty acids as the source of the C_6-unit.[50] These results and monitoring the stereochemical course of the enoylreductase steps during formation of the hexanoyl unit,[51] all pointed to either a dedicated fatty acid synthase (FAS) to prepare the unusual C_6-primer for homologation by a polyketide synthase (PKS), or a sophisticated polyprotein capable of both reductive and nonreductive malonyl chain extention of acetate to norsolorinic acid (**5**).

Resolution of this issue has emerged from the recent localization of an ∼60 kb gene cluster that likely encodes the entire aflatoxin biosynthetic pathway. Identification of this cluster in *A. parasiticus* was uncovered by the development of a transformation system effective in this organism by Linz[52,53] and complementation of the classically-obtained mutants *ver-1* and *nor-1*.[54,55] In parallel with these experiments, the *S*-adenosylmethionine-dependent *O*-methyltransferase (OMT-A) responsible for the conversion of sterigmatocystin (**15**) to *O*-methylsterigmatocystin (**16**) was purified to homogeneity[56] and its corresponding gene cloned.[57] This gene, *omtA*, and the two genes identified by complementation were localized to a 45 kb region of genomic DNA.[58] Purification and characterization of versicolorin B synthase (VBS), which catalyzes the closure of (±)-versiconal (**11**) to (−)-versicolorin B (**12**),[46,47] led to the cloning of its encoding gene and its localization just 3.3 kb downstream in the cluster from *omtA*.[59] Sequencing the DNA flanking these genes has led to the identification of a number of probable open reading frames (ORFs) including a divergently transcribed pair of FAS genes (*fas-1A* and *fas-2A*), highly homologous to the yeast FAS β- and α-subunit genes, respectively, and a separately transcribed PKS.[60] This structural organization is consistent with the first FAS-PKS model outlined above and has received experimental support that the proposed roles of these genes are indeed as suggested.[61]

Scheme 2

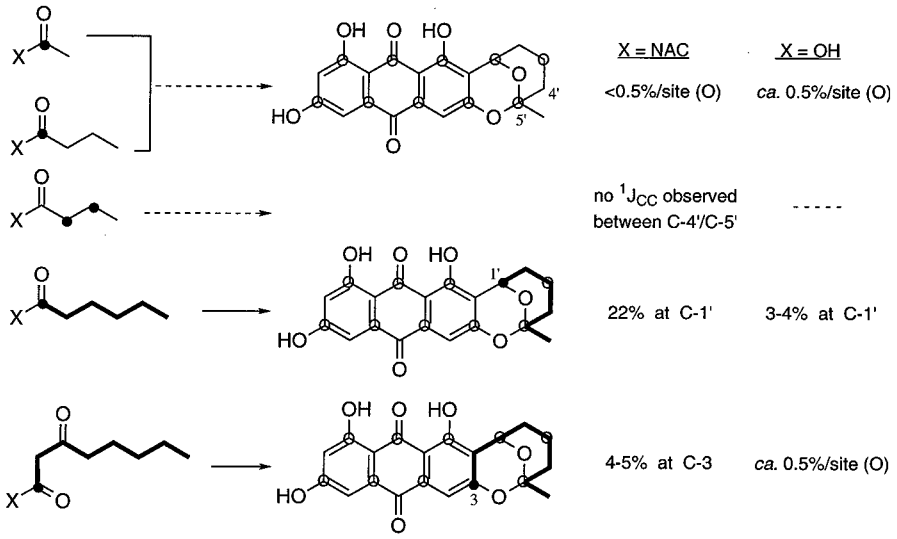


Meticulous chemical degradation experiments of Büchi *et al.* established the acetate/polymalonate origins of aflatoxin,[18,19] and the use of classically obtained mutants of *A. parasiticus* defined a series of anthraquinone intermediates in the first half of the pathway (orange to red pigments). In contrast, conventional biochemical approaches to the problem using cell-free methods and attempted purifications of biosynthetic enzymes have yielded comparatively meager returns for the effort invested.[46,62] In this article, we review the published enzymological studies of the aflatoxin pathway and the rapid emergence of genetic methods in the last half dozen years.

II. Molecular Biology and Genetics of the ST/AFB$_1$ Pathways

A comprehensive description of the ST/AFB$_1$ pathways should be founded upon both chemical and biological aspects which include reaction mechanisms for each transformation, an understanding of its genetic organization and metabolic regulation, and ideally, the answer to the question: "What purpose do the pernicious toxins produced by certain *Aspergillus* species serve?" A recent series of molecular biological investigations has begun to unravel both structural and regulatory elements of the ST/AFB$_1$

Scheme 3

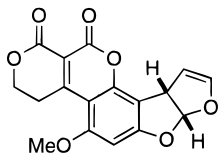


biosynthetic pathways, advancing toward resolution of the above issues. A practical aim of these studies is the abolition of carcinogen production by the excision of key regions of the *Aspergillus* genome through recombinational inactivation, particularly in commercial species such as *A. sojae*, which is used in soya sauce fermentations. For agricultural applications, a biocompetitive approach in which an atoxigenic strain of *Aspergillus* displaces the native organism may be envisioned.[60,63]

Four methods have been employed to characterize the ST/AFB_1 pathways on the levels of both genes and enzymes including: (a) complementation of classically generated mutants, (b) targeted disruption of suspected pathway genes in wild-type species, (c) purification of biosynthetic enzymes explicitly for the isolation of coding sequence through degenerate codon-derived oligonucleotide primers ("reverse genetics") and, to a minor extent, (d) random mutagenesis. The overall goals of these endeavors include the purification and characterization of individual biosynthetic enzymes to unravel the cryptic transformations of this pathway and cloning of the genes regulating and encoding the biosynthetic pathway to allow the control or eradication of the deterimental effects of AFB_1. In the following sections, the methods and techniques employed are described and insights gained for the individual chemical transformations depicted in Scheme 2 are reviewed.

A. Recombination Experiments in *Aspergillus* Species

The isolation and characterization of *Aspergillus* strains produced by ultraviolet light or chemical random mutagenesis to define the AFB_1 pathway was initiated by Lee and Bennett and relied upon the chromophoric properties of AFB_1 and the pathway intermediates.[20,24] The B ("blue") and G ("green") series aflatoxins are substituted coumarins that are highly fluorescent under long-wavelength ultraviolet light. Zones of blue-fluorescent AFB_1 are secreted into coconut agar medium around aflatoxigenic, wild-type *A. parasiticus* colonies.[64] *A. flavus* produces aflatoxins B_1 (**1**), B_2 (**20**), G_1 (**21**), and G_2 (**22**), whereas *A. parasiticus* and *A. nomius* afford



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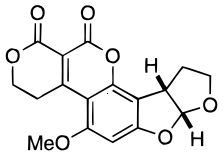

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mainly AFB_1 and some AFB_2. Colonies from mutated spores had reduced or undetectable aflatoxin production occasionally coupled with the accumulation of the yellow to orange-red anthraquinone pigments NA (**5**), AVN (**6**), AVR (**8**), HVN (**9**), or VA (**13**) which facilitated colony screening. A recent advance in the use of random mutagenesis was the development of a judicious phenotype-based colony selection by combining UV photography with a small volume "tip culture" method to screen for colonies with an impaired ability to synthesize aflatoxins.[65]

Advances in recombinant DNA technology and the difficulty of generating new biochemically characterized *A. parasiticus* mutants propelled researchers toward methods that target specific genetic loci through genetic transformation and recombination to restore a mutant to a wild-type phenotype or to generate desired "knockout" strains.

The Linz and the Woloshuk/Payne groups developed protoplasting methods for *A. parasiticus*[52,53] and *A. flavus*,[66] respectively, whose resistance to transformation initially hindered studying the molecular biology of these organisms. However, digestion of the cell wall allowed the passage of exogenous DNA into the host cell via polyethylene glycol plus Ca^{2+}-induced cell fusion.[67,68] As no mitotically stable replicons have been identified in aflatoxigenic *Aspergillus* species, insertion by homologous recombination of the selectable marker-containing construct into the genome is required for the propagation of transformed DNA (Figure 1). Typical markers confer benomyl (fungicide) resistance (*bml*)[69−72] or nitrate (*niaD*)[52,73] or uracil (*pyrG*) prototrophy.[53,66,71]

Single-crossover experiments with circular plasmid or cosmid DNA containing a complete wild-type copy

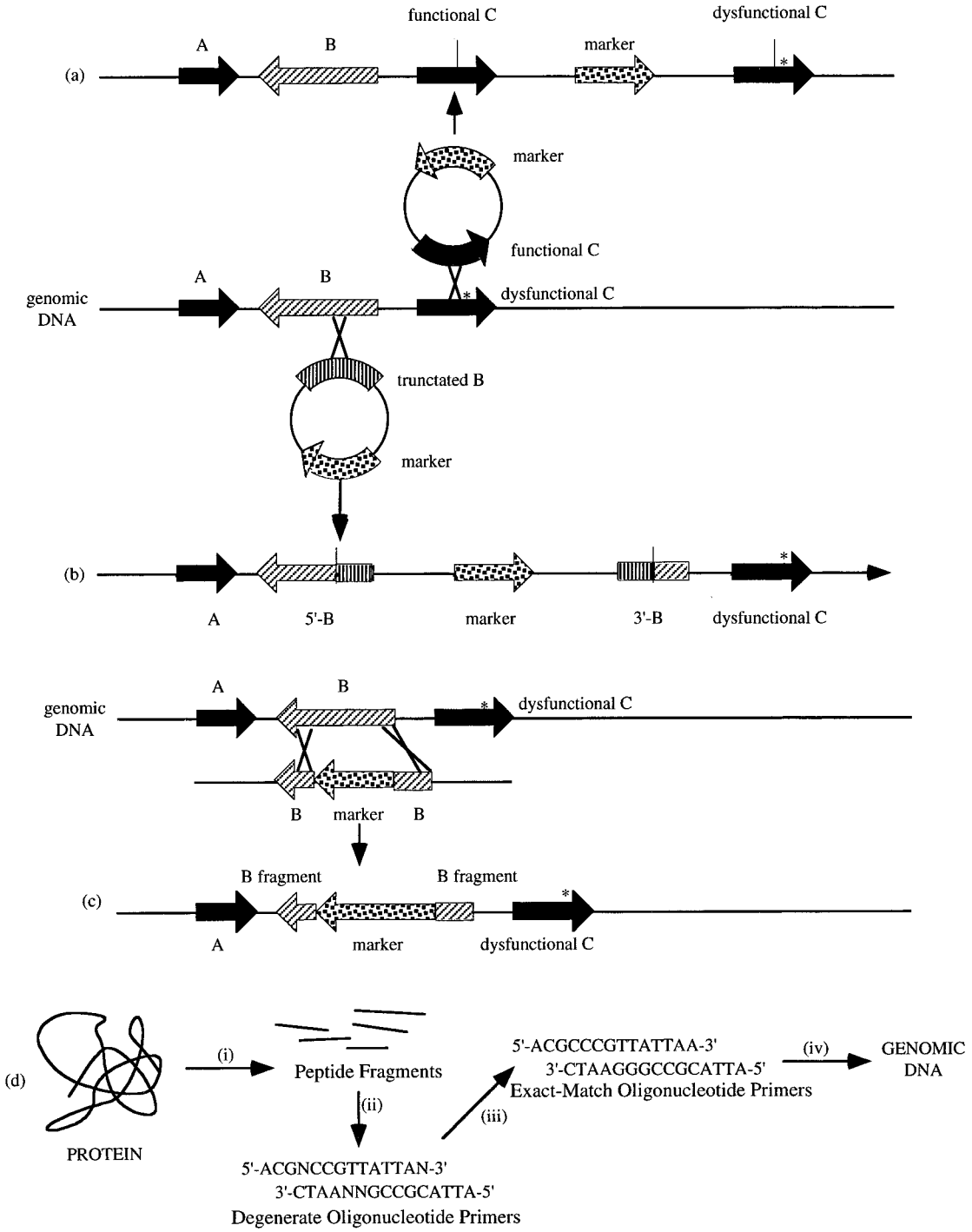


Figure 1. (a) Complementation of a dysfunctional gene C with a functional gene copy from an exogenous, circular plasmid. Mutations are denoted with an asterisk. (b) Recombinational inactivation, or gene disruption, of a functional gene B. Transformation of cells with a 5′- and 3′-truncated fragment of gene B on a circular vector followed by homologous recombination with chromosomal DNA results in two truncated, dysfunctional gene fragments. (c) In a similar experiment to b, use of a linearized plasmid containing a selectable marker inserted within the sequence of gene B requires two crossover events to maintain a viable chromosome; this results in a "one-step gene replacement" of an internal portion of B. Insertional junctions are marked by vertical bars. Experimentally, appearance of activity from biosynthetic gene product C or loss of gene B function is observed by the respective processing or accumulation of the substrates. Inclusion of a selectable marker facilitates screening of transformed cells. (d) The process of reverse genetics. A protein is isolated, (i) partially digested by protease(s) providing fragments for peptide sequencing. (ii) The typically degenerate nucleotide sequences which can be deduced from the protein sequence are used (iii) as probes in the PCR or hybridization screening of a cDNA library. The exact sequence of the primer is obtained by sequencing clones from initial screening, which may be subsequently employed in (iv) the screening of genomic libraries. The progression is from protein to messenger RNA (cDNA) to genomic DNA.

of a defective gene installs the vector and yields a functional chromosomal copy of the targeted gene (Figure 1a). This method, termed gene complementation, has ultimately led to the cloning of the *afl-2* gene from *A. flavus*[74] and *nor-1*,[54] *ver-1*,[55] and *apa-2*[75] from *A. parasiticus*. It should be noted that recombination leading to complementation of the mutant phenotype may also occur at heterologous

sites (ectopic integration).[76,77]

In contrast, the use of a 5′- and 3′-truncated gene in a single crossover experiment causes the insertion of the foreign DNA, selectable marker and vector resulting in specific gene inactivation at the homologous genomic site (gene disruption) and potentially blockage of the biosynthetic pathway (Figure 1b). Finally, linearized exogenous DNA requires a double-crossover event for site-specific integration and gene replacement to occur. During this process the replacement of a segment flanked by two regions homologous to the chromosomal DNA occurs (Figure 1c). Depending upon the experimental design, this method can be used as a "one-step gene replacement" or for site-directed mutagenesis. Gene disruption has uncovered seven genes, including *nor-1*[54] and *pksA*[78] from the AFB_1 pathway and *stcA*,[79] *stcL*,[80] *stcP*,[81] *stcS*,[82] and *stcU*[83] from the ST pathway.

In reverse genetics, the isolation of a native protein provides amino acid sequence which is used in the design of oligonucleotide probes of the corresponding genomic sequence (*i.e.* in practice, translation to transcription to genome). Reverse genetics has the appeal of targeting structural genes corresponding to proteins identified by an *in vitro* chemical assay. Although the quantity and stability of the aflatoxin-synthesizing enzymes has been an obstacle, this method has led to the identification of three pathway genes: *omtA*,[57] *vbs*,[59] and *norA*.[84]

B. Clustering of Pathway Genes

Organization, including both the order and the direction of transcription, of clustered genes has regularly been suggested to be important in the efficient, coordinated formation of secondary metabolites. Compact genomic organization is common in prokaryotes,[85] however, it has been only sporadically reported in higher organisms. Examples of eukaryotic structural genes known to be localized include four ethanol metabolism genes of the *alc* regulon which are clustered on chromosome VII of *A. nidulans*,[86] as well as the fungal genes for penicillin,[87] melanin[88] and trichothecene toxin biosynthesis,[89] nitrate utilization,[90] and quinic acid metabolism.[91]

Initially, researchers believed that *Aspergillus* structural genes were distributed throughout the genome, as occurs in most eukaryotes.[92] Papa determined that the aflatoxin-biosynthesis-related mutations *afl-1* and *afl-2* were unlinked in *A. flavus* by parasexual analysis.[93] Two other mappable mutations, *afl-4* and *afl-b2*, were found in linkage groups II[94] and VIII.[95] Lennox *et al.* reported that the AFB_1-linked genes were distributed between six or seven linkage groups.[96] Reproduction by a parasexual cycle, which allows recombination via heterokaryon formation, has presented the major genetic difficulty in studying *A. parasiticus* and *A. flavus* in comparison to the model organism *A. nidulans*.

Later research has shown conclusively that many of the structural genes for aflatoxin biosynthesis are tightly clustered. Despite his earlier work, Papa assigned nine mutations, including *norA* and *afl-1*, to linkage group VII.[97,98] The initial direct evidence for physical linkage was provided by Linz and coworkers who reported that two genes, *nor-1* and *ver-1*, were proximate through the hybridization of restriction fragments to a cosmid.[55] Parasexual analysis of Afl^- mutants in *A. parasiticus* had given inconsistent results concerning the linkage of *nor-1* and *ver-1*.[99,100]

Restriction mapping of lambda and cosmid clones from *A. parasiticus* and *A. flavus* containing *omtA* provided a 60 kb physical map encompassing nine genes, including *nor-1*, *ver-1*, *aflR*, *pksA*, and *omtA* (Figure 2).[58] The observation that the genes for the entire AFB_1 pathway are ostensibly at a single locus on the chromosome was a major experimental breakthrough enabling researchers to uncover new genes by targeting regions neighboring previously described genes. The genes *ord-1* (renamed *avnA*), which is a putative cytochrome P450 monooxygenase, and *ord-2* (no assignable homology) were identified immediately downstream of *omtA*.[58] Although *ord-1*, *ord-2*, and *omtA* are closely spaced and colinear, no evidence for cotranscription was observed.[101] The right side of the cluster was expanded beyond *omtA* by the mapping of clones neighboring the gene *vbs*.[59] The experimentally determined distance between *vbs* and *omtA* was 3.3 kb, with an intervening cytochrome P450 monooxygenase gene.

The *afl-1* locus in *A. flavus* possesses a 120 kb deletion including most of the AFB_1 biosynthetic cluster.[102] Complementation with three cosmids containing 90 kb of *A. flavus* DNA restores AFB_1 biosynthesis.[103]

Hybridization experiments denoted the presence of a second copy of the *ver-1* gene in wild-type *A. parasiticus* SU-1.[104] The predicted amino acid sequence for the coding regions showed 95% identity between the genes; however, a nonsense mutation was present at codon 87 which strongly suggests that only one copy is functional. The duplicated region extends 12 kb upstream of the renamed functional *ver-1A* and dysfunctional *ver-1B* genes in *A. parasiticus*. It has been observed that for *A. flavus*, which does not contain the duplication, only 40% of the strains produce AFB_1.[104−106] Southern blotting indicated two copies of the *norA* gene may be present in the *A. parasiticus* genome, although the location of the second copy is unknown.[84] This gene may correspond to *aad*[58] which is in the duplicated region from *ver-1A* to *aflR*.

The sterigmatocystin pathway is expected to require each cognate enzyme from the entire AFB_1 pathway, excepting at least one protein following the formation of ST (**15**) (Scheme 4). A contiguous 60 kb region of chromosome IV in *A. nidulans* in the region of *verA* encompassed 24 actively transcribed regions including homologs for *vbs*, *nor-1*, *norA*, *aflR*, *ver-1*, *pksA*, and *fas-1A*.[107] Although only the functions of *stcA*, *stcJ*, *stcK*, *stcL*, *stcP*, *stcS*, and *stcU* have been rigorously assigned, other open reading frames encode four cytochrome P450 monooxygenases, a peroxidase, and an FAD monooxygenase gene which are expected to be involved in many of the cryptic oxidative structural transformations between norsolorinic acid (**5**) and sterigmatocystin (**15**). Notably absent is the cognate gene for *omtA* required for the penultimate conversion in the AFB_1 pathway. The presence of *stcT* and *ord-2* in the clusters, which

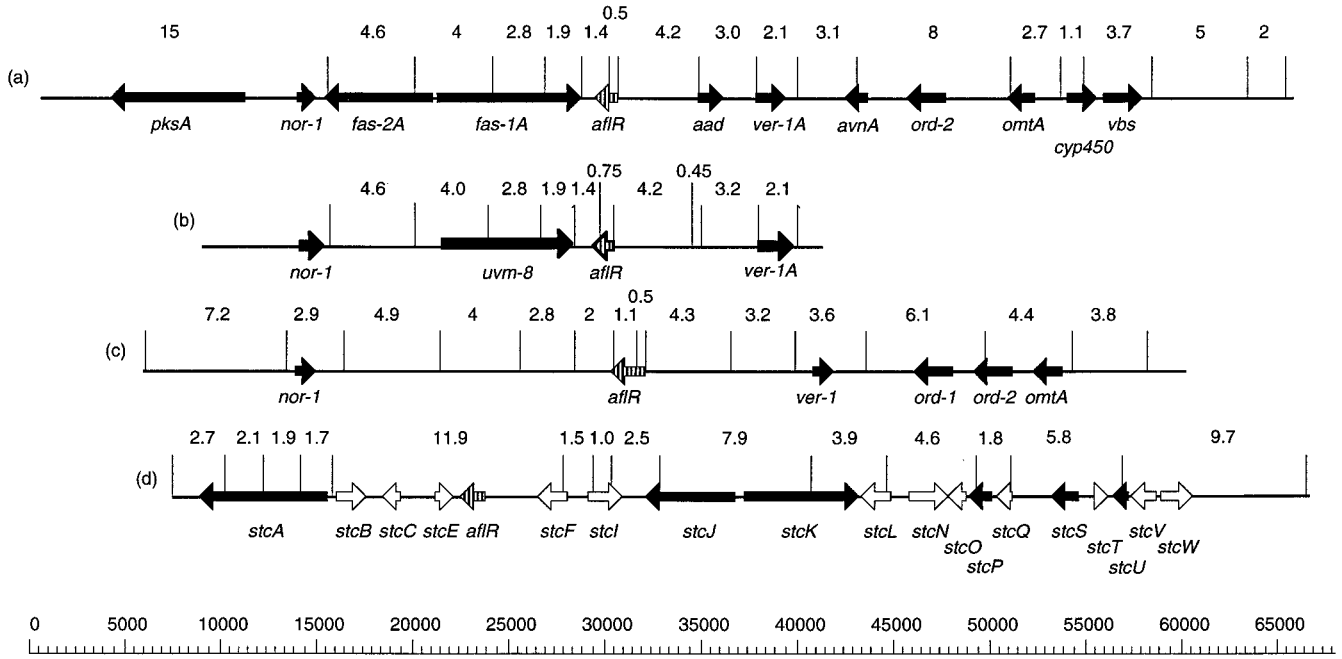


Figure 2. Aflatoxin biosynthetic gene clusters observed in (a) *A. parasiticus*, (b) *A. parasiticus* NorA genomic cosmid clone, (c) *A. flavus*, and (d) the sequenced sterigmatocystin biosynthetic gene cluster from *A. nidulans*. *Eco*RI restriction sites are shown by vertical bars and corresponding fragment sizes are listed above the sequence (in kb). Genes whose function has been experimentally supported are indicated in black arrows. Function based upon homology is denoted by open arrows and regulatory genes are marked by barred arrows. Experimentally supported functions are shown in Scheme 4. Gene designations, listed as groups of homologs (former names underscored), are *aflR*, *apa-2*, *afl-2*, zinc binuclear cluster motif regulatory gene; *omtA*, *omt-1*, *omt-1b*, *O*-methyltransferase; *ord-2*, monooxygenase; *stcA*, *pksST*, *pksA*, *pksL1*, polyketide synthase; *stcB*, P450 monooxygenase; *stcC*, peroxidase; *stcE*, *nor-1* ketoreductase; *stcF*, *avnA*, *ord-1*, P450 monooxygenase; *stcI*, lipase/esterase; *stcJ*, *fas-1A*, fatty acid synthase (β-subunit); *stcK*, *fas-1B*, fatty acid synthase (α-subunit); *stcL*, P450 monooxygenase; *stcN*, *vbs*, versicolorin B synthase; *stcO*, oxidoreductase; *stcP*, *O*-methyltransferase; *stcS*, *apstcS*, *verB*, P450 monooxygenase; *stcT*, elongation factor 1γ; *stcU*, *verA*, *ver-1a*, ketoreductase; *stcV*, *norA*, *aad*, dehydrogenase/reductase; and *stcW*, FAD monooxygenase.

resemble translation elongation factor γ, remains an enigma. Southern hybridization to electrophoretically separated *A. nidulans* chromosomes placed *stcU*, and therefore the whole *stc* cluster, at the left end of the 2.9 Mb chromosome IV.[83]

The presence of seemingly closely related biosynthetic pathways in the phylogenically distant organisms *Bipolaris*, *Chaetomium*, *Farrowia*, and *Monocillium* is suggestive of a horizontal gene-transfer event.[108] Parallels between certain fungal genes involved in melanin and spore pigment biosynthesis and certain ST/AFB_1 pathway genes as well as the general prevalence of introns supports their archaic eukaryotic origins. Localized physical similarities are present between *A. flavus*/*A. parasiticus* and *A. nidulans* (Figure 2).[58] However, substantial "unit-like" reorganization is apparent which may affect individual gene regulation. Amino acid identity encoded among the genes of the AFB_1-producing species is commonly >90%, whereas in *A. nidulans* often less than 80% similarity is retained.

C. Structural Genes and Enzymes Involved in ST/AFB_1 Biosynthesis

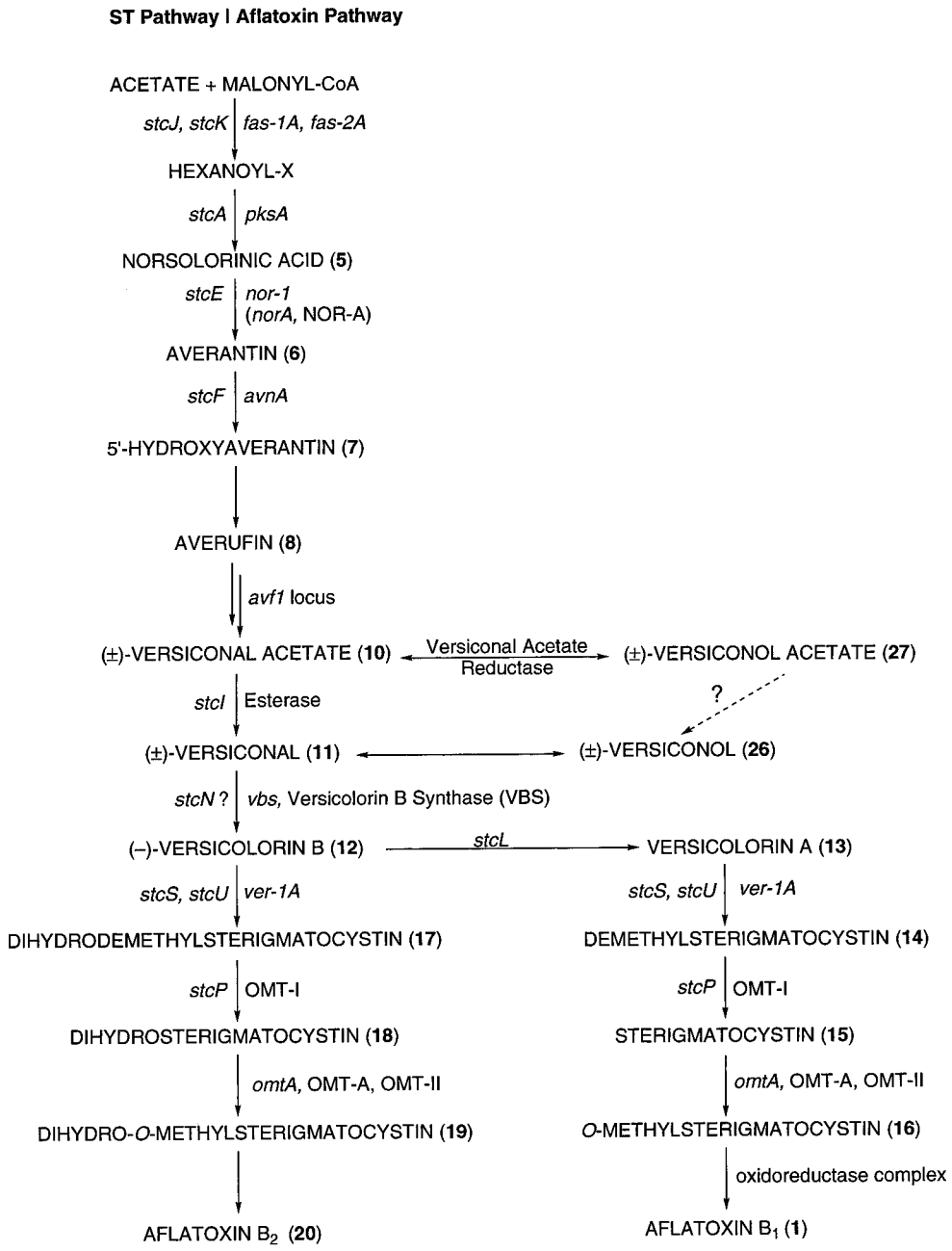
1. Fatty Acid and Polyketide Formation

The formation of the linear polyketide **4** occurs in two phases beginning with the formation of a hexanoyl starter unit by a fatty acid synthase, which is subsequently extended by a polyketide synthase.

Ultraviolet mutagenesis of a norsolorinic acid-accumulating *A. parasiticus* mutant strain yielded a double mutant, UVM8 (*fas-1A*, *nor-1*).[109] Transformation of UVM8 with a plasmid subclone of the NorA genomic DNA cosmid (shown in Figure 2)[101] complemented specifically the *fas-1A* locus reestablishing norsolorinic acid (NA, **5**) synthesis. Sequence of the subclone provided a new open reading frame, *fas-1A*, which was transcribed as a single 7.5 kb mRNA. Identification of FAS/PKS genes has been aided by widely conserved sequences within their functional domains. Regions with 58 and 69% amino acid similarity to the enoyl reductase and malonyl/palmitoyl transferase domains of yeast fatty acid synthase FAS1 (β-subunit),[110] respectively, and insertional inactivation experiments strongly suggested the functional requirement for *fas-1A* in hexanoyl primer formation (see below).[109]

Distinct fatty acid synthase genes involved in primary and secondary metabolism have been identified in *A. nidulans*.[79] The genes *stcJ* and *stcK* are specifically required for the formation of the hexanoyl starter unit of ST (chromosome IV), whereas long-chain fatty acid synthase subunits *fasA* and *fasB* necessary for cell growth are found on chromosome VIII. The gene products from *stcJ* and *stcK* contain three and five domains, respectively, analogous to the FASα and FASβ genes required for fatty acid biosynthesis in *Saccharomyces cerevisiae*.[111,112] The translational initiation sites for the divergently tran-

Scheme 4



scribed genes are separated by 495 bp and have identical sequence in the −2 to +5 region. Similarity of start regions has been suggested to control the subunit stoichiometry for the *S. cerevisiae* multimeric complex.[112] The similarity of chemical function between *Penicillium* and *Yarrowia* primary metabolic FASs and the putative hexanoyl-generating FAS is not, however, maintained in the overall 44 and 37% amino acid identity of the *stcJ* and *stcK* sequences to the FAS protein sequences.

A. parasiticus mutants produced by insertional inactivation of *fas-1A* in norsolorinic acid-accumulating strains converted hexanoylNAC to NA (**5**) with unexpectedly low efficiency, even though the *fas-2A* and *pksA* genes (see below) were demonstrated to be intact.[61] An efficient 39% incorporation of hexanoylNAC into **5** was verified in control experiments[61] and agreed with earlier studies.[50] Minimal effects by β-oxidation inhibitors were noted in incorporation experiments with these *A. parasiticus fas-1A* disruption mutants.[61] We interpret these data to signify a substantial channeling interaction between the PKS and FAS subunits. In partial support of this idea, the overall yield of ST (**15**) from analogous *A. nidulans* FAS mutants grown on hexanoic acid-enriched medium is 20-fold lower than the wild-type strain.[79]

Certain aspects of *Aspergillus* development are affected by the levels of secondary metabolites. A 9-fold increase in the number of sclerotia (nutrient storage/survival structures composed of compact hyphal masses) was observed in *fas-1A* disruptants when compared to mutant strains accumulating AFB_1 pathway intermediates, but this difference was less marked on comparison with wild-type strains which produced AFB_1.[109]

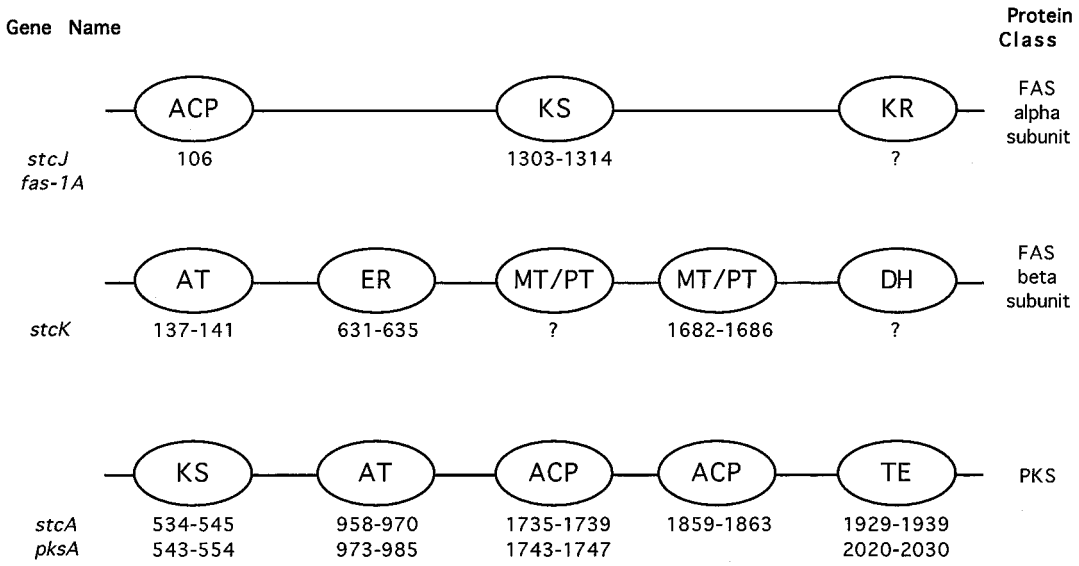


Figure 3. Pictorial representation of the fatty acid synthase subunits and the polyketide synthase involved in the early steps of ST/AFB_1 biosynthesis. Approximate locations of the sterigmatocystin fatty acid synthase active sites for each cognate domain to the yeast FAS α and β subunits are indicated where possible, as based upon local amino acid sequence homology. Abbreviations: ACP, acyl carrier protein; KS, β-ketoacyl synthase; KR, β-ketoacyl reductase; AT, acyl transferase; ER, enoyl reductase; MT/PT, malonyl/palmitoyl transferase; DH, β-hydroxyacyl dehydratase; TE, thioesterase.

2. Hexanoyl Unit to Norsolorinic Acid

The ST and AFB_1 PKS genes responsible for the nonreductive elongation of the hexanoyl starter unit to polyketide **4** have been found to encode large type I multifunctional enzymes characteristic of most eukaryotes.[113,114] The polyketide synthase gene *pksA*/*pksL1* from *A. parasiticus* was isolated independently by two research groups in 1995.[78,115] Transcriptional analysis of the 35 kb NorA cosmid identified 14 mRNAs, eight of which were cotranscribed with *nor-1* and *ver-1*.[101] Three large transcripts (6.5, 7.0, and 7.5 kb) were noted among these, including *fas-1A*. Partial sequence of the *pksA* gene, corresponding to the 7.0 kb transcript, showed 80% amino acid similarity to the β-ketosynthase and acyl transferase regions of the 9.2 kb *wA* gene of *A. nidulans*.[116] The initial yellow polyketide produced by WA,[117] which is structurally related to emodin and norsolorinic acid, is converted to the conidial pigment by the laccase encoded by *yA*.[118,119] Independent disruption of the *pksA* locus in an *O*-methylsterigmatocystin-accumulating strain of *A. parasiticus* with the plasmid pXX, which contained a truncated *pksA* gene, resulted in an albino strain incapable of producing NA (**5**).[78] The absence of the enoyl reductase and β-ketoacyl reductase domains necessary for saturated fatty acid biosynthesis is fully in keeping with the nonreductive polyketide homologation of the hexanoyl starter unit by the polyketide synthase.[78]

Differential hybridization screening, a method that allows the identification of genomic DNA clones by comparing the ability of mRNA isolated from organisms grown under two or more sets of conditions to hybridize to the genomic DNA,[120] was used by Leonard and co-workers to identify the 6.6 kb *A. parasiticus pksL1* mRNA (renamed *pksA*).[115] Nineteen genomic clones were selected by identifying those that hybridized to *pksA* cDNA, prepared from poly(A)$^+$-mRNA isolated from cultures in permissive and nonpermissive medium at different growth phases, in a manner correlating to AFB_1 production. The calculated 2109 amino acid translated sequence contained four functional domains ordered acyl carrier protein (ACP), β-ketoacyl synthase (KS), acyltransferase (AT), and thioesterase (TE), but no β-ketoacyl-ACP reductase or enoyl-ACP hydrase domains (Figure 3). Cotransformation of an *afl*$^+$, *niaD*$^-$ *A. parasiticus* strain with a *niaD*$^+$marker plasmid and a linearized plasmid containing a 4.5 kb genomic fragment of *pksL1* resulted in three *afl*$^-$, *niaD*$^+$ mutants whose Southern hybridizations were consistent with insertional inactivation at the expected locus. No intermediates involved with the AFB_1 pathway were isolable from either the culture broth or the mycelia of the disrupted strains.

Later, Yu and Leonard isolated several cosmids from an *A. nidulans* genomic library that both hybridized to a *nor-1* cDNA fragment and mapped a 55 kb deleted region in two non-ST producing random mutants.[121] Transcriptional mapping of these cosmids revealed a 7.2 kb transcript for the *pksA* homolog, *stcA* (originally named *pksST*), and a neighboring gene (*stcB*) with similarity to the cytochrome P450 monooxygenase superfamily. Numerous polyadenylation sites in *stcA* were distributed over 150 nt although the biological relevance of 3′-end heterogeneity, which is commonly observed in plants, is yet to be uncovered. The 2181 amino acid *stcA* gene product contained a β-ketoacyl synthase, acyltransferase, two proximal ACP domains, and thioesterase.

The overall similarity of the STC-A protein sequence to PKS-A and WA was found to be 77% and 65%, respectively,[120,121] which raises the possibility of interchanging polyketide synthases between *Aspergilli*. Structurally, the PKS genes *wA* and *pksA* appear to be more closely related than *pksA* and *stcA*. Both the *pksA* and *wA* genes were fragmented by five introns compared to two in the sterigmatocystin PKS.[115,121,122] The insertion points for the first two introns in *wA*, *PKS1*, and *pksA* are conserved.

In addition to *stcA*, three other PKSs with duplicated ACP domains—*wA* (*A. nidulans*), *pksX* (*Bacillus subtilis*), and *PKS1* (*Colletotrichum lagenarium*)—may be cited.[116,123,124] Although transfer of the starting alkanoyl unit from the FAS to the PKS may seem to be the likely ACP function, the enzyme PKS1 uses an acetate starter unit. Further biochemical studies will be required to elucidate the use of this seemingly superfluous domain.

Consensus sequences for the β-ketoacyl-ACP synthase (GP----DTACSS-L), acyltransferase (G--P----GHS-G--A), acyl carrier protein (D/ELG-DSL), and thioesterase (GPY---G-S-G) were clearly present in each domain. The consensus sequence for a thioesterase active site, which is absent in 6-MSA synthase, strongly resembles that of the serine esterases (G-X-S-X-G) and appears to be a universal motif for this emerging subclass of polyketide synthase domains. It is conceivable that the thioesterase serves to release the hexanoyl thioester starter unit from either the fatty acid ACP or an intermediate CoA ester or, alternatively, is involved in the cyclization and product release of a PKS-bound octaketide intermediate to yield the anthrone. It is noteworthy that a distinct dehydratase/cyclase domain, which has been reported in both type I and II PKSs,[125−127] has not been found in any *Aspergillus* sequence.[121] The quaternary interactions between the FASα/FASβ/PKS subunits, which will be key to unraveling the initial transformations, are yet to be understood.

The selectivity of the PKS toward primer size and structure has been briefly examined through feeding experiments with the norsolorinic acid-accumulating mutant of *A. parasiticus* NOR-1.[128] The NAC thioesters of pentanoic acid and 6-fluorohexanoic acid were successfully incorporated to lead to modest yields of modified norsolorinic acids, while butanoyl-, heptanoyl-, and octanoylNAC were not utilized under these experimental conditions.[128]

3. Norsolorinic Acid to Averantin

The *n*orsolorinic *a*cid *r*elated gene *nar-1*, renamed *nor-1*, was the first gene identified by complementation of a NA-accumulating, nitrite-reductase-deficient *A. parasiticus* mutant with a cosmid from a wild-type genomic library.[54] Northern blotting identified a 1.4 kb transcript that hybridized to the *nor-1* fragment, which was further characterized by gene disruption. One-step gene replacement of the *nor-1* gene produced blocked strains that accumulated NA (**5**) and demonstrated a significantly reduced ability to produce AFB_1.[129] Although leakage was observed in all transformants, homologous recombination was localized to a short-chain alcohol dehydrogenase-like sequence that together with the biochemical results provide strong, direct support for the involvement of the 29-kDa *nor-1* gene product as the principal dehydrogenase responsible for the conversion of norsolorinic acid (NA, **5**) to averantin (AVN, **6**) in the biosynthetic pathway.[130]

One of perhaps several alcohol dehydrogenases of minor biosynthetic significance has been identified from cell-free studies where reduction of the benzylic ketone of NA was found in early experiments to require either NADPH or NADH.[131−133] Correspondingly, cell-free experiments with *A. parasiticus* demonstrated that $NADP^+$ and NAD^+ were capable of driving this equilibrium toward the oxidized species **5**.[133] Further purification revealed a monomeric NADPH-dependent protein with a molecular mass of approximately 40 kDa and optimal activity at pH 7.5 and 30 °C.[134,135] Monoclonal antibodies (MAbs) were prepared to a partially purified 43 kDa protein band with norsolorinic acid reductase activity.[84,136] Time-dependent Western analysis of crude *A. parasiticus* extracts using the MAb revealed a single 43 kDa band appearing after 36 h in an aflatoxin-permissive medium.[84] Immunochemical screening yielded a single cDNA clone encoding a 388 reisdue, 43.7 kDa protein. Northern analysis showed that transcription was concurrent with expression. The gene identified was denoted *norA* and corresponded to *aad* previously sequenced just downstream of *ver-1* (see below). Both aryl alcohol dehydrogenase (49% amino acid homology to *Phanerochaete chrysosporium*)[137] and NADPH-binding motifs were identified in the translated nucleotide sequence. However, the *norA* gene product showed little homology to alcohol dehydrogenases generally, and shows only 22% amino acid identity to the dehydrogenase translated from *nor-1*. While addition of the MAb to a crude cell-free extract resulted in 60% reduction of NA reductase activity,[136] disruption of *norA* in *A. parasiticus* did not result in the accumulation of pigmented intermediates or reduced levels of AFB_1 production.[84] So, although *norA* maps to the aflatoxin gene cluster, its significance in the pathway to the mycotoxin is uncertain. In this connection Southern blot analysis of genomic *A. parasiticus* DNA suggested the possible presence of a second copy of *norA*.

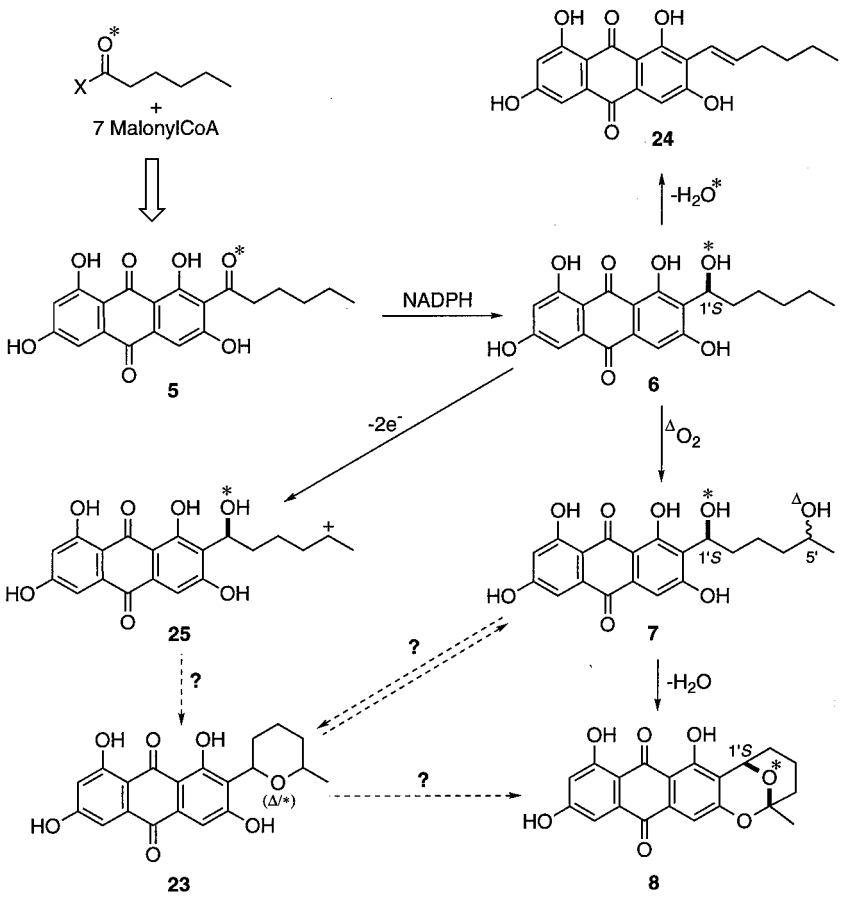
In other independent work, affinity chromatography of a cell-free extract resulted in a 138-fold purification of a norsolorinic acid reductase of unreported size.[131,138] A fourth enzyme capable of the reduction of **5** to **6**, optimally at pH 8.5 and 35 °C, has been reported by Dutton.[138] This 140 kDa species was isolated by sequential Reactive Green-19 agarose and norsolorinic acid affinity columns. The K_ms for **5** and **6** were 3.45 and 3.72 μM, respectively; hence, feedback inhibition may exert metabolic control at this step. A slight preference for NADPH (K_m 103 μM) over NADH was also noted.

4. Averantin to Averufin

The conversion of averantin (**6**) to averufin (AVR, **8**) is surrounded by controversy. Averufanin (**23**) has been isolated from a number of *Aspergillus* sp. including *A. flavus* and *A. versicolor*.[139−141] McCormick *et al.* have reported that [^{14}C]averufanin, obtained from the uptake of [1-^{14}C]acetate in a blocked mutant, incorporated radioactivity into AFB_1 in wild-type *A. parasiticus* and into versicolorin A (VA, **13**) and averufin (AVR, **8**), but not into averantin (AVN, **6**) in the appropriate blocked mutants.[142] These experiments would suggest, therefore, that **23** lies on the biosynthetic pathway between **6** and **8** (Scheme 5).

In opposition, however, Yabe and co-workers have isolated aflatoxins when the UV-irradiation-derived *afl*$^-$ mutant of *A. parasiticus* NIAH-26 was fermented

Scheme 5



in the presence of **5**, **6**, 5′-hydroxyaverantin (**7**, isolated from the filamentous fungus *Emericella heterothallica*) or **8**.[133] A cytosolic fraction derived from this mutant converted **6** to a diastereotopic mixture of 1′*S*,5′*S*- and 1′*S*,5′*R*-diols **7**. By comparison, the 1′*R* enantiomer of **8** was inactive under these conditions. Importantly, when **23** or averythrin (**24**) were incubated with NIAH-26, no aflatoxins could be detected.[133,135] Prieto *et al.* have observed averufanin buildup in averufin-accumulating *avf-1* mutants which is relieved following complementation of the *avf1* locus.[103] Therefore, these findings clearly point to averufanin as a shunt product, probably derived by cyclization of **7** or **25**, while further oxidation to the 5′-ketone would spontaneously and rapidly cyclize to the internal ketal **8**.

Yu *et al.* isolated a single averantin-accumulating transformant resulting from a double-crossover event with an 8.2 kb *niaD*-containing fragment interrupting the *avnA* locus.[143] Genomic sequence correlated to an open-reading frame whose calculated translation yields the 495 amino acid, 56.3 kDa AVN-A protein. Sequence analysis identified several conserved cytochrome P450 motifs including helix I, believed to delineate the oxygen-binding pocket, the highly conserved heme-binding region (F--Gpr-CIG), and the hydrophobic amino-terminal region absent in initial reports.[58] Feeding of anthraquinones prior to averantin resulted in no production of aflatoxin; however, intermediates later in the pathway, including 5′-hydroxyaverantin (**7**) and averufanin, led to the formation of AFB_1 and AFG_1.

It is difficult to find a rationale that accommodates the opposing points of view about the role of averufanin. The absolute configurations of AVN (**6**)[144] and AVR (**8**)[145] are both 1′*S*, set during the reduction of **5**. In averantin, the C-1′ oxygen is derived from the polyketide precursor.[146] To satisfy these experimentally determined preconditions, either the cyclization of **7** must occur with loss of the secondary C-5′ alcohol, or a hypothetical intermediate in the 5′-oxidation of averantin itself (*e.g.*, cation **25**) is directly cyclized to give averufanin-containing polyketide oxygen at the C-1′/5′ bond (*O). The isolation of a second enzyme requiring averufanin either through genetic or biochemical experiments or, alternatively, determining the source of the averufanin 1′*S* oxygen will ultimately resolve this quandry.

5. Averufin to Versiconal Acetate

Of the six carbons present in the linear side chain of AVR, four remain in the dihydrobisfuran of versicolorin A (**13**) and AFB_1 (**1**). Specific labeling experiments have unambiguously determined that the outer intact acetate unit is severed from the C_6 side chains of the anthraquinone intermediates norsolorinic acid (**5**) and averufin (AVR, **8**)[39,40,147] and subsequently released by an esterase as acetate.

No detailed information is available concerning the rearrangement of the linear hydrocarbon side chain of averufin to the branched chain of the bisfurans in which the migrating C-1′ center changes from the alcohol to the aldehyde oxidation state without loss of the directly bound hydrogen.[41,43,51,141,145,148,149] How-

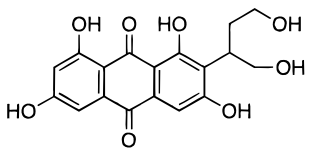
ever, chemical model reactions mimicing this process have been carried out.[150,151] A 7 kb genetic locus *avf1* was found during the complementation of an *A. flavus* mutant which has been reported to mediate the AVR (**8**) to versiconal acetate transformation.[103]

The terminal acetate unit in versiconal acetate (**10**, Scheme 2) is derived from a Baeyer−Villiger-like rearrangement process with no detected exchange of the acetate with the metabolic pool.[38] The substrate for this rearrangement, 1′-hydroxyversicolorone (**9**), was isolated from a new mutant of *A. parasiticus*[23] and, when prepared in isotopically labeled form, was demonstrated to give the expected specific labeling pattern in AFB_1 for intact incorporation.[43]

6. Versiconal Acetate to Versiconal

Hsieh[152] and Dutton[153,154] independently reported cell-free systems from *A. parasiticus* capable of carrying out the conversion of versiconal acetate (**10**) to versicolorin A (VA, **13**). Both of these investigators observed that the overall transformation was blocked by Dichlorvos, an insecticidal esterase inhibitor, and was reduced in efficiency by anaerobic conditions. Subsequently, the activity of a versiconal-releasing esterase in cell-free extracts was demonstrated.[44,155] Three enzymes capable of hydrolyzing **10** have been recently identifed by anion-exchange chromatography of 55 h *A. parasiticus* cell-free extracts.[156] One of these species was partially purified in five steps and has been determined to be a dimer of 32 kDa subunits. The gene *stcI* has been proposed to encode the esterase activity in the *A. nidulans* gene cluster.[107]

Versiconol (**26**) has been isolated in an optically active form from *A. versicolor*.[157] The role of this metabolite, as well as its corresponding ester **27**, has been examined by Yabe and co-workers.[158−160] Con-



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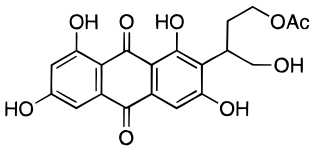


27

version of the racemic substrate versiconal acetate through the metabolic grid of oxidations and reductions was shown by Yabe to provide a low 2:1 predominance of the 2′*R* enantiomer for each of the reduced intermediates and, consequently, cannot be the transformation which locks the 2′*S* stereochemistry found in the aflatoxins.[158] Two versiconal acetate reductase activities that catalyze this step have been found in the cytosolic fraction of *A. parasiticus* mutant NIAH-26 and have been purified and characterized.[160]

7. Versiconal to Versicolorin B

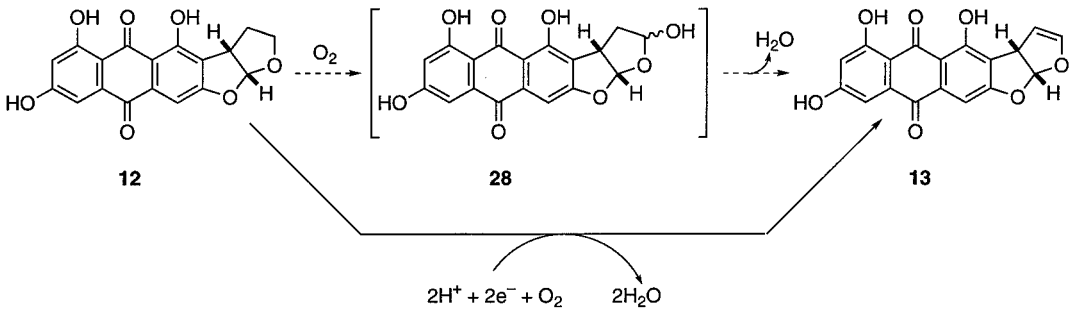
The dehydrative cyclization of racemic versiconal (**11**) to (−)-versicolorin B (VB, **12**) is catalyzed by versicolorin B synthase, VBS, to reestablish asymmetry in the aflatoxin biosynthetic pathway.[44,47,158,161] While averantin (**6**) and averufin (**8**) are formed in optically pure form,[144,145] the furan hemiacetal-containing intermediates **9**−**11** are isolated as racemates.[23] The tetrahydrobisfuran cyclizing enzyme proved difficult to purify, nonetheless two isoelectric focusing steps, seizing upon the remarkably broad pH-stable activity range (pH 4−8) and the pI = 4.7 ± 0.1 of VBS, greatly improved the efficiency of earlier isolation attempts.[46,161] Chromatography then on Mono Q provided homogeneous protein, which was found to be active as a dimer of identical 78 kDa subunits.[47] An earlier purification reported a subunit molecular mass of 72 kDa.[161]

It had been observed in preliminary cell-free experiments that *all* versiconal acetate (**10**) could be converted to (−)-**12**, suggesting that either (a) VBS could cyclize both enantiomers of **11** to (−)-**12**, perhaps by catalyzing the enantiomerization process itself, or (b) the inherent chemical rate of racemization was sufficiently fast that VBS simply selects the correct antipode of versiconal (**11**) from solution and rapidly converts it to (−)-versicolorin B (**12**). Detailed kinetic analyses revealed that VBS does not affect the rate of racemization and that the protein had a high affinity for its substrate ($K_m = 1\ \mu M$) compared to that typically found for biosynthetic enzymes.[47] The critical rate of racemization could not be determined by optical or NMR methods, but was finally measured in a series of kinetic experiments in which the ratio of enzyme to substrate was varied until the rate of enzymic cyclization became first order, dependent on the intrinsic rate of racemization. This rate constant was determined to be 0.9 s^{-1} while under V_{max} conditions the turnover number was 2.5 s^{-1}. By taking into account inhibition by the product and the catalytically unreactive substrate stereoisomer, the dynamics of cyclization and racemization could be completely modeled. The outcome of these experiments shows that racemization is unlikely to restrict significantly, if at all, the metabolic flux to versicolorin B (**12**).[47]

The *N*-terminal sequence from LysC-endopeptidase digested VBS was essential for the preparation of partially degenerate oligonucleotide primers.[59] A 750 bp *vbs* gene fragment was PCR-amplified from a 60 h *A. parasiticus* cDNA library yielding coding sequence which was in agreement with the extended peptide sequence not used for primer generation. A PCR-generated 615 bp internal fragment was then used for cDNA and gDNA library hybridization screening. The isolated 1932 bp open reading frame was broken by a single 53 bp intron in the genomic clone. Translation of the *vbs* coding sequence yielded a 643 amino acid, 70.3 kDa protein.

Examination of the protein sequence revealed a 58% overall similarity to glucose oxidase from *A. niger* and other members of the glucose-methanol-choline (GMC) flavin oxidoreductase family.[59] Strikingly, although the FAD binding (G-G--G) and active-site motifs for these enzymes are retained in VBS, the cyclase does not carry out a redox reaction or require flavin for activity.[47] It is believed that one function of FAD in the GMC family is to fix the tertiary structure in an active, properly folded conformation.[162−165] Yet, an apparent 23 amino acid gap exists within the FAD binding site of VBS, which

Scheme 6



may eliminate flavin binding. Furthermore, several residues believed to provide important stabilizations for the nucleotide phosphate moiety in glucose oxidase (H78, T30)[166] are absent in VBS (Y102, A52).

Several plant-derived mandelonitrile lyases, also members of the GMC family based upon conservation of primary structure, catalyze nonredox, hydrolytic processes and do not bind flavin.[167,168] The apparent biochemical relationship between mandelonitrile lyase isolated from *Ximenia americana* and VBS suggests that other aspects of the molecular architecture may be pertinent. The lyase is a glycoprotein with a low p*I* of 3.9 that stereospecifically hydrolyses a single substrate enantiomer and is specific for aryl-substituted nitriles.[168] Comparing wild-type VBS before and after PNGase F treatment uncovered extensive *N*-glycosylation of the native protein.[169] Overexpression in *Saccharomyces cerevisiae* has been optimized in a vector incorporating a yeast secretion signal which provided VBS indistinguishable from the native protein in a 50−100 times higher yield compared to *Aspergillus*.[169] This method for the preparation of glycosylated enzymes, such as VBS, will undoubtably facilitate detailed biochemical studies of other posttranslationally modified proteins important in the AFB_1 pathway and confer advantages of ease of isolation and uniformity of posttranslational glycosylation.[170]

8. Versicolorin B to Versicolorin A

The conversion of versicolorin B (VB, **12**) to versicolorin A (VA, **13**) lies at the critical juncture between the dihydrofuran- and tetrahydrofuran-containing aflatoxin series. The partitioning of labeled samples of **12** and **13** into AFB_1 and AFB_2 supported a simplified view of bisfuran formation and the precursor relationship of tetrahydro- to dihydrobisfurans early in the biosynthesis.[46,47,171]

Direct pathways for the net oxidation of **12** include initial oxidation to versicolorin A hemiacetal (**28**) followed by dehydration or the direct desaturation of **12** (Scheme 6). Incubation of **28** in the presence and absence of oxidized or reduced nicotinamide cofactors resulted exclusively in reduction to the tetrahydrobisfuran **12**, implying that a desaturation step must occur to convert **12** to **13**.[46,47,172] Shortly thereafter, Yabe described the "desaturase", an NADPH-dependent enzyme present in the microsomal fraction of *A. parasiticus*.[173]

The *A. nidulans* gene *stcL* has recently been insertionally inactivated which blocked the oxidative branch of the pathway resulting in the accumulation of dihydrosterigmatocystin (**18**).[80] The predicted 512 amino acid protein contained the distinctive heme-binding site found in all fungal cytochrome P450 monooxygenases. As conventional hydroxylation has been excluded in the experiments above, the reaction catalyzed by this oxygenase may be an example of a two-electron oxidation without incorporation of oxygen in the substrate.[41,150,151,174] A gene highly homologous to *stcL* has been identified downstream of *avnA* in *A. parasiticus*.[175] A defect in the desaturase would account for the observation that natural isolates[176] and mutagenized strains[177] of *A. flavus* produce AFB_2 but little or no AFB_1. Furthermore, the proportions of AFB_1 series to AFB_2 series metabolites would be controlled by the relative activities of the desaturase and the initial dihydrodemethylsterigmatocystin (DHDMST, **17**) to dihydrosterigmatocystin (DHST, **18**) *O*-methyltransferase.

9. Anthraquinone to Xanthone Transformation

The postbisfuran part of the biosynthetic pathway involves considerably less well-understood nuclear cleavage and rearrangement processes leading first to the variously *O*-methylated sterigmatocystins **14**−**16** and **17**−**19** and then to the substituted coumarins of AFB_1 (**1**) and AFB_2 (**20**). The absence of isolatable intermediates has obscured the apparently multistep conversion of the anthraquinone versicolorin A (**13**) to the xanthone demethylsterigmatocystin (DMST, **14**), a net transformation involving oxidative cleavage, saponification, nuclear rearrangement, reduction, and decarboxylation.[178−180] Molecular biology has elucidated several of the genes involved, providing a framework for testing potential synthetic intermediates.

Transformation of a VA-accumulating strain of *A. parasiticus* with a genomic library from aflatoxigenic *A. parasiticus* yielded a single Afl^+ transformant.[55] Genomic DNA isolation from the transformed cells followed by digestion with a restriction enzyme and recircularization allowed the recovery, or "marker rescue", in *E. coli* of the complementing *ver-1* DNA. Mapping of transcripts with fragments of the isolated DNA revealed a 1.0 kb mRNA. The corresponding genomic DNA contained an open reading frame fragmented by two introns 50 and 61 bp in length with canonical GT···AG boundaries. The predicted 262 amino acid polypeptide showed 52% similarity to two NADPH-dependent polyketide ketoreductases[181,182] and a polyhydroxynaphthalene reductase involved in melanin biosynthesis, including the amino-

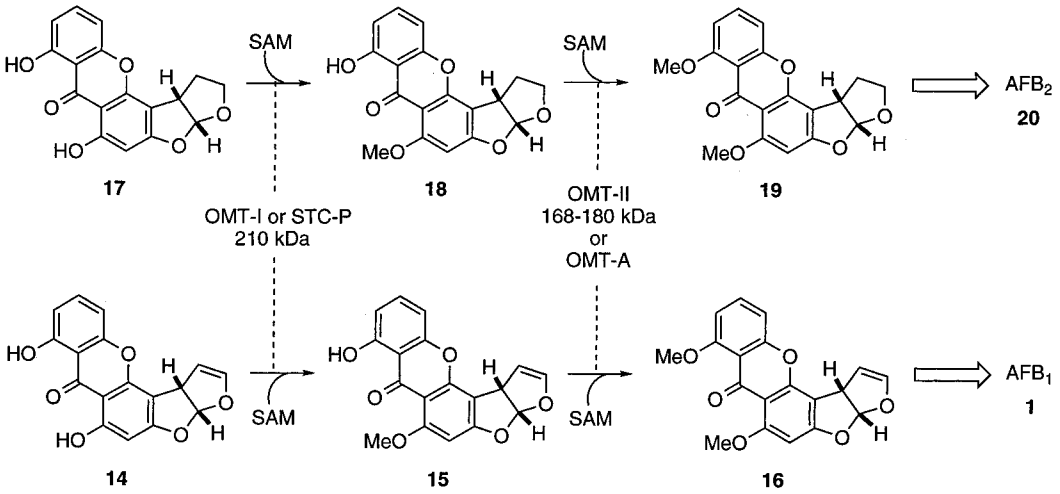


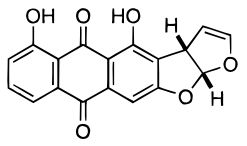
Figure 4. Parallel dihydrofuran and tetrahydrofuran pathways involving xanthone:*S*-adenosylmethionine methyltransferases. Enzymes are linked by dashed lines to favored transformations. The gene product from *stcP* is believed to catalyze the first methylation in *A. nidulans*. The function *in vivo* of OMT-A remains to be clarified.

terminal adenine nucleotide binding motif (G-G--A----------K).[183] Sclerotia production was dramatically increased by the removal of the genetic block at *ver-1*, providing a link between late-growth development and secondary metabolism.[55]

The control of the *ver-1* gene, which was transcribed primarily during idiophase, was examined in greater detail using β-tubulin and *ver-1A* promoter constructs with the β-glucuronidase (GUS) reporter gene.[184] The profile of transcript accumulation for the construct was similar to the wild-type *ver-1* gene when fused to the 1.1-kb *ver-1A* 5′-region. However, relocation of the construct to the *niaD* locus reduced GUS transcription to 1/500th wild-type levels while maintaining the shape of the time course. The control promoter for the housekeeping β-tubulin gene resulted in uniformly high levels of transcription. The influence of positional effects on gene expression is potentially an important factor controlling AFB_1 levels.

The functions of the *stcS* and *stcU* structural genes for sterigmatocystin (ST, **15**) formation in *A. nidulans* have been partially assigned by disruption experiments.[82,83] Disruption of either gene resulted in the accumulation of versicolorin A (**13**) and nearly complete suppression of ST formation. *stcU*, formerly designated *verA*, possesses an extended 85% amino acid identity with *ver-1* in *A. parasiticus*.[83] This putative NADPH-dependent ketoreductase may be necessary for the reduction at the C-6 phenolic carbon prior to decarboxylation. An *stcL* and *stcU* doubly disrupted mutant accumulated versicolorin B (**12**), which specifically demonstrated the requirement of *stcU* for the conversion of **12** to **17**.[80] Genomic sequence analysis of *stcS* (formerly *verB*) indicated that this gene encodes a 505 amino acid cytochrome P450 monooxygenase, which is sufficiently unique to classify it in a new P450 family.[82]

The conversion of **13** to **15** illuminates a primary difficulty in studying the complex transformations within the overall pathway: many intermediates appear to be unstable or enzyme bound. A known *Aspergillus* metabolite, 6-deoxyversicolorin A (**29**)[185] was prepared in isotopically labeled form to test directly whether the aromatic reduction at C-6 initiates the reaction sequence. Anthraquinone **29**



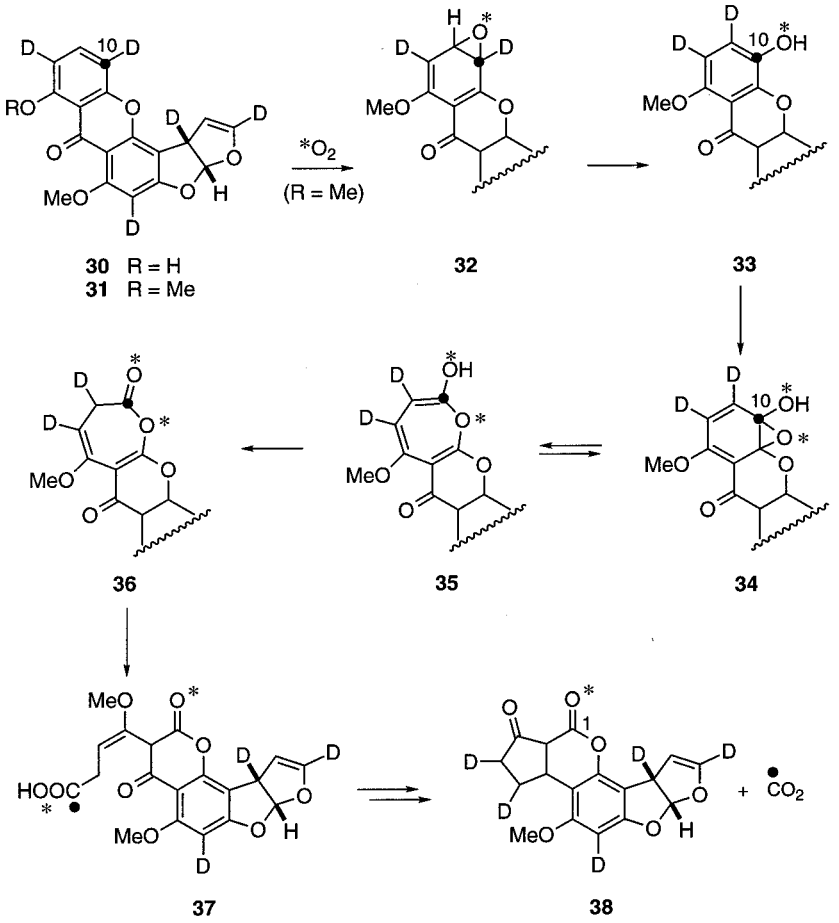
29

was not converted into AFB_1 under two distinct experimental protocols, whereas **15** was efficiently utilized under comparable conditions.[186] Hence, it was concluded that reduction is not the first step. Other attempts to determine the functional order of the STC-S and STC-U enzymes have failed as neither disruption mutant nor cross-fed cultures produced visible intermediates or **15**.[82] Clever approaches to tackling the problems of protein−protein interaction will be essential for the solution of this problem and, for example, in characterizing a large oxidoreductase complex reported to carry out the ultimate conversion of *O*-methylsterigmatocystin (OMST, **16**) to AFB_1.[171,187]

10. Xanthone Methyltransferases

A monomeric 40 kDa methyltransferase, OMT-A, that catalyzes the conversion of ST (**15**) to OMST (**16**) (Figure 4) was isolated in five steps by Keller.[56] The K_ms for **15** and *S*-adenosylmethionine (SAM) were detemined to be 2.0 and 9.6 μM, respectively. A 24 h cDNA library was successfully screened using polyclonal antibodies specific to the purified protein and resulted in the isolation of the 1254 bp coding sequence for *omt-A* (formerly named *omt-1*).[57,188] Comparison of native *N*-terminal sequence with the translated 5′-terminal coding sequence indicated that a 41 amino acid segment was absent from the N-terminus of the originally isolated 377-residue protein. The hypothetical function of the hydrophobic leader is to promote membrane association,[57] although other functions may be envisioned. The polyclonal antibodies also recognized an overexpressed OMT-A-β-galactosidase fusion protein which was capable of the SAM-mediated conversion of **15**

Scheme 7



to **16**. The conservation of the single-copy *O*-methyltransferase genes between *A. parasiticus* and *A. flavus* was evident from the >97% nucleotide identity in the coding sequence, as well as the identical lengths and locations of four introns.[188]

A second heterodimeric cytosolic enzyme composed of 58 and 110 kDa subunits has been purified by anion-exchange chromatography.[171,189] Partial characterization of the enzyme demonstrated the requirement for SAM and a K_m of 1.8−2.0 μM for **15**. Competition experiments indicated a preference for ST (**15**) over dihydrosterigmatocystin (DHST, **18**, Figure 4). This complex was shown to be distinct from the 40 kDa species by *N*-terminal sequence, molar absorptivity, and its affinity for SAM.[56] Antibodies raised against this enzyme weakly cross-reacted with the previously identified 40 kDa species.

Two distinct methyltransferase activities were also observed by Yabe in an AFB_1-permissive medium (Figure 4).[190] *O*-Methyltransferase I, estimated to be 210 kDa in size by gel filtration chromatography, was proficient in the conversion of DHDMST (**17**) and DMST (**14**) to DHST (**18**) and ST (**15**), respectively. The 180 kDa *O*-methyltransferase II processed DHST (**18**) and ST (**15**) to dihydro-*O*-methylsterigmatocystin (DHOMST, **19**) and OMST (**16**), respectively. The type II activity may correspond to the previously reported 168 kDa complex.[189] The two enzyme complexes were further distinguished by the specific *N*-methylmaleimide inhibition of the type I complex. The evidence provided by both research groups is consistent with distinct enzymatic activities sequentially converting **14**−**16** to AFB_1, and the tetrahydrobisfuran intermediates to AFB_2.

Disruption of the *A. nidulans stcP* gene, which is presumed to be involved in transfer of the first methyl group, resulted in the accumulation of demethylsterigmatocystin (**14**).[81] The predicted protein sequence from this gene only exhibited 30% identity with the *omtA* gene.

11. Coumarin Formation—Formation of AFB_1 and AFB_2

Molecular biological and biochemical studies of the oxidative ring cleavage and concomitant nuclear rearrangement in the aflatoxin pathway are very sparse. An NADPH requirement has been observed for the reaction of *O*-methylsterigmatocystin (OMST, **16**) to AFB_1, implying the involvement of an NADPH-dependent monooxygenase.[187,191] Evidence has been gathered that an ~200 kDa protein complex concentrated in the microsomal fractions of the *A. parasiticus* mutant strain AVN-1 catalyzes this transmutation.[171] This species, which has been purified 150-fold in five steps, exhibited a preference for the dihydrofuran substrates as shown by competition experiments (apparent K_ms of **16** and **19**, 1.2 and 13.4 μM, respectively).

Several lines of indirect evidence have been gathered that constrain the possible mechanism that can be acting in the final steps of the biosynthesis: (a) The ^{14}C label at C-10 in OMST (**31**, Scheme 7) is ultimately lost as carbon dioxide in the formation of aflatoxin.[192] (b) Incorporation of oxygen from molec-

ular oxygen ($^{18}O_2$) occurs at C-1 with an efficiency comparable to that at other sites in the mycotoxin,[193] and complementary to those sites labeled in sterigmatocystin from [1-^{13}C,$^{18}O_2$]acetate.[194] (c) In earlier experiments of Sankawa[148] and Simpson[195] where [$^2H_3$]acetate had been incorporated into sterigmatocystin (**30**) and AFB_1 (**38**), respectively, an "NIH shift" was noted in the conversion of the former to the latter.[195]

One potential mechanistic route that accommodates these observations is shown in Scheme 7. A monooxygenase-catalyzed epoxidation of **31** to **32** would be consistent with the known NADPH dependence of the overall reaction, and account for the NIH shift of deuterium label evident in the cyclopentenone ring of AFB_1 (**38**). No experimental evidence exists for 10-hydroxy-*O*-methylsterigmatocystin (**33**) proposed in this mechanism, but further oxidation (illustrated here as an additional monooxygenase step) to **34** and opening to the oxepin **35** might be expected to tautomerize to the labile lactone **36** and hydrolyze. Closure, decarboxylation, and demethylation can then be visualized to give aflatoxin B_1 (**38**).

A gene from *A. flavus* reportedly involved in the conversion of *O*-methylsterigmatocystin (**16**, Scheme 2) to AFB_1 named *ord1* (not to be confused with *ord-1/avnA* described above[143]) has been localized to a 3.3 kb region.[103]

D. Regulation of ST/AFB_1 Formation

Time course experiments to monitor the appearance of AFB_1 as well as the transcripts and the gene products of *nor-1* and *ver-1* during idiophase indicated that partial regulation of AFB_1 biosynthesis occurs at the transcriptional level.[196,197] In nearly all instances examined, similar patterns of transcript accumulation have been reported for the AFB_1-related genes; a single exception, however, lies with the strongly conserved regulatory factor gene *aflR*, previously named *afl-2* (*A. flavus*) and *apa-2* (*A. parasiticus*). In *A. nidulans*, mRNA for *aflR* was visible after 24 h and peaked after approximately 72 h growth, a period that fully brackets that of the other *stc* genes examined.[198] The *aflR* genes in *A. flavus* and *A. parasiticus* exhibited nucleotide sequence identity in excess of 95%.[75,199] Hybridization of *afl-2* to similar sequences in *A. parasiticus*, *A. oryzae*, and *A. sojae* has been observed, although the gene is often absent in the AFB_1 nonproducing strains.[200]

The *A. parasiticus aflR* gene, located 8 kb from *ver-1*, was initially cloned by the identification of NOR-1 and VER-1 strains which overproduced pathway intermediates NA (**5**) and VA (**13**) (Figure 1), respectively, following transformation with a cosmid library.[75] Transformants with two copies of the regulatory gene were shown to have increased enzyme activity for norsolorinic acid and sterigmatocystin metabolism,[75] whereas mutants resulting from the inactivation of *aflR* in *A. nidulans* were incapable of transcribing the ST-specific mRNAs for *stcW*, *stcV*, *stcU*, and *stc T*.[198] Complementation of the *A. flavus afl-1* strain to an aflatoxigenic phenotype was found to require *aflR*.[103] *A. flavus aflR* was capable of regulating ST production in transformed *A. nidulans* cells demonstrating the interchangeability of *aflR* homologs.[198]

The 46.7 kDa protein possesses a zinc cluster motif (Cys--Cys------Cys------Cys----Cys------Cys) found in other fungal transcriptional regulatory proteins.[201−204] The *N*-terminal region surrounding the zinc cluster presents a high 71% identity over 42 amino acids, compared to an overall 33% identity between homologs in *A. nidulans* and *A. flavus*/*A. parasiticus*.[198] The function of two moderately conserved carboxyl-terminal regions (44 and 35% identity over 84 and 52 amino acids, respectively) has yet to be determined but a role in binding to a negative regulator of the pathway has been suggested.[205] Several strongly conserved nucleotides in the 5′-nontranslated region of this gene have been considered as important taxonomic markers.[200]

An overexpressed AFLR fragment has been shown to have a weak affinity for GAL4 promoter-like regions (5′-TTAGGCCTAA-3′) upstream of *aflR* in *A. parasiticus*.[205] However, this sequence does not occur within the ST gene cluster and is rarely present preceeding AFB_1 biosynthetic genes. This binding site may allude to *aflR* acting as a autoregulatory *trans*-acting factor implying that AFB_1 biosynthesis is positively regulated in a similar fashion to sulfur and nitrogen metabolism in *A. nidulans* and *N. crassa*.[206−210] To further demonstrate the ability of *aflR* to positively regulate ST biosynthetic genes, the *aflR* coding sequence from *A. flavus* was inserted downstream of the *A. nidulans niiA* promoter. Shifting from ammonium- to nitrate-containing medium resulted in the induction of *aflR* under the control of the *niiA* regulatory domain. Transcription of *aflR* was accompanied by the rapid appearance of *stcU* mRNA within 2 h, and several other ST-related transcripts within 8 h compared to 24 h for the normal time course of *stc* gene transcription.[198] Interestingly, the antisense transcript *aflRas*, which may also play a regulatory role, has also been observed.[199] The exact molecular mechanism of *aflR*-mediated regulation remains to be determined.

Strong stimulatory and inhibitory effects of ammonium and nitrate ions, respectively, provide a substantive link between nitrogen and AFB_1 regulation.[205,211−213] Other factors which affect AFB_1 production include zinc, cytosolic NADPH/$NADP^+$ levels, and carbohydrate sources.[196,214−219]

Multivariate analysis determined that maximal yields of aflatoxins from *A. flavus* occur on YES (yeast extract−sucrose) agar at 30 °C and a water activity of 0.996 after 15 days.[220] Hypothetical defensive roles for aflatoxin may be complemented by other fungal toxins, such as cyclopiazonic acid which is coproduced by certain isolates of *A. flavus* but was found to plateau under independent conditions.[221] The influence of environmental factors and evolutionary changes has been suggested to construct a viable chemical defense system for the local ecology of *Aspergillus* species.

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