Purification and Characterization of Versicolorin B Synthase from *Aspergillus parasiticus*. Catalysis of the Stereodifferentiating Cyclization in Aflatoxin Biosynthesis Essential to DNA Interaction†

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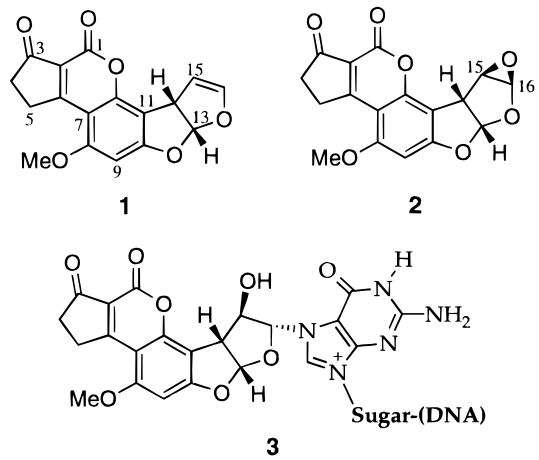
ABSTRACT: The absolute configuration of the dihydrobisfuran ring system characteristic of aflatoxin B_1 is essential to the covalent reaction of its metabolically activated form with double-stranded DNA. The biosynthesis of this potent mycotoxin proceeds through three configurationally labile intermediates to racemic versiconal hemiacetal. Subsequent enzymatic cyclization establishes the stereochemistry of this critical ring fusion in (−)-versicolorin B and is catalyzed by versicolorin B synthase (VBS). The isolation and purification of VBS from *Aspergillus parasiticus* (SU-1, ATCC 56775) and its kinetic characterization and attempted inactivation are described. Initial purification trials were plagued both by a chromophoric impurity which was difficult to remove and by low recoveries of active protein. The discovery of a remarkably broad pH range of enzyme stability and catalytic activity led to an efficient procedure involving preparative isoelectric focusing and ion exchange FPLC chromatography. The enzyme behaved as a dimer upon gel filtration and migrated with M_r 78 000 Da during denaturing gel electrophoresis. The UV spectrum of pure VBS gave no evidence of a bound chromophore. Detailed kinetic analysis of VBS revealed that this protein selects from two equilibrating enantiomers of versiconal hemiacetal to cyclize the appropriate antipode to optically pure versicolorin B. By varying the amount of enzyme to a fixed concentration of substrate, the rate of enzymic cyclization could be limited by the intrinsic rate of enantiomerization of the substrate under the conditions of reaction. It was possible to quantitate the dynamics of this substrate enantiomerization/cyclization process, to establish the role played by VBS, and to evaluate the significance of each to the overall biosynthesis of aflatoxin. The potential role of an acidic residue of the enzyme in catalysis was supported by analysis of the pH−rate profile of VBS and chemical labeling studies. Successful demonstration of competitive inhibition of VBS by a simple substrate analogue led to the design and synthesis of a potential mechanism-based inactivator of the protein.

Aflatoxin B_1 (**1**, Chart 1) is a potent environmental carcinogen produced by *Aspergillus flavus* and *Aspergillus parasiticus*, common molds that infect nuts and grains and pose a significant threat to the food supply world-wide (Dickens, 1977; Lillehoj & Hesseltine, 1977). The origin of its toxic effects to humans has been intensively investigated and is understood to involve oxidative activation by cytochromes P-450, notably in the liver (Aoyama et al., 1990; Forrester et al., 1990), to afford the *exo*-epoxide **2**. Generation of this species *in vivo* has been inferred from the structure of its covalent adduct with DNA (Gopalakrishnan et al., 1990) and has been firmly established by unambiguous chemical synthesis and its separate reaction with DNA (Baertschi et al., 1988; Iyer et al., 1994). The epoxide **2**, while short-lived in protic media (Baertschi et al., 1988), is relatively lipophilic and intercalates readily into double-stranded DNA to give selective adduct formation through the N-7 of guanine residues (Essigmann et al., 1977; Loechler et al., 1988). A direct link has been forged between the metabolic activation of aflatoxin and the incidence of human cancers by the discovery of a mutational "hot spot" in the p53 gene highly favored for reaction with epoxide **2**. A transversion (G249C) has been found to occur frequently as a consequence of depurination during repair at this site to give an amino acid change (Arg → Ser) in the ultimately translated protein (Aguilar et al., 1993; Hsu et al., 1991). The p53 protein normally takes part in regulation of the cell cycle (Harper et al., 1993; Harris & Hollstein, 1993; Yonish-Rouach et al., 1993), but this mutant is defective in this role, allowing selective clonal expansion of the affected cells (Aguilar et al., 1993; Harris, 1991; Hsu et al., 1991).

Chart 1



1 **2** **3**

The binding and selective reaction of metabolically-activated aflatoxin at guanine residues depend intimately on

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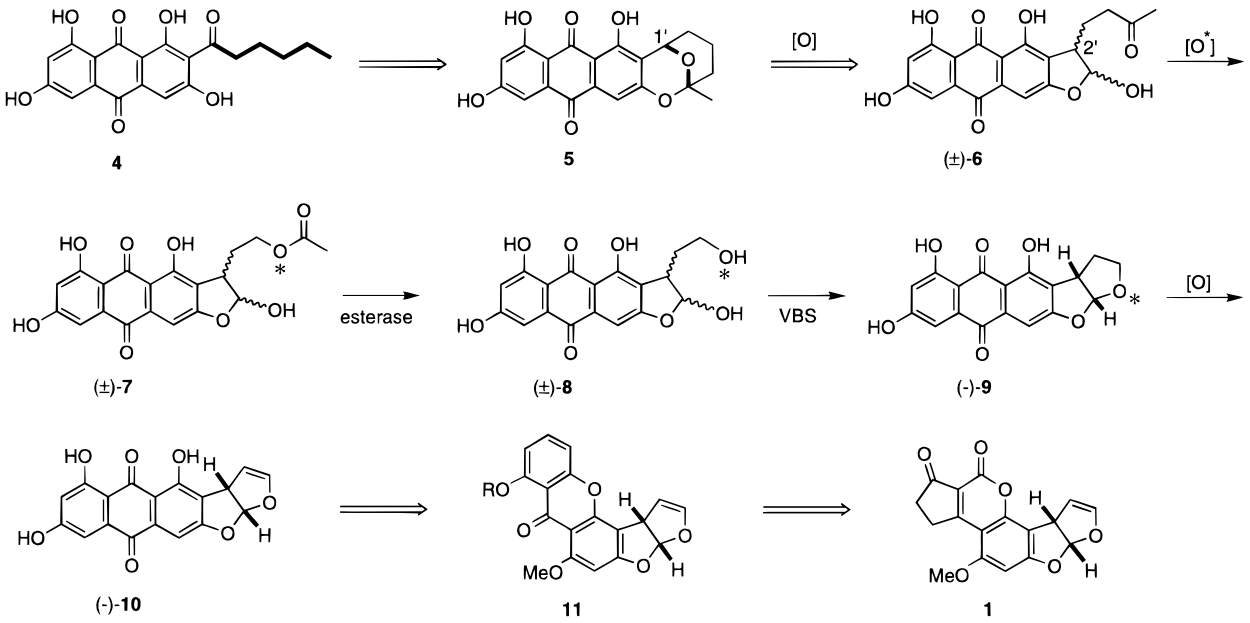
Scheme 1



the absolute configuration of the dihydrobisfuran uniquely characteristic of this family of polyketide metabolites (Iyer et al., 1994). Detailed ^{1}H NMR analyses of this adduct in short oligonucleotides have shown that the mycotoxin stacks to the 5′-side of the modified guanine. This orientation presents the *exo*-epoxide of **2** for reaction with guanosine in the major groove to give adduct **3** in which the cyclopentenone ring hydrogens extend into the minor groove (Gopalakrishnan et al., 1990). While the DNA is unwound to accommodate the oxidatively activated aflatoxin, the helix remains right-handed and is slightly perturbed from B-form only in the immediate region of the covalent reaction (Gopalakrishnan et al., 1990). A very similar picture has been derived for the corresponding adduct of sterigmatocystin (**11**, R = H) (Gopalakrishnan et al., 1992). Thus, reaction of the *exo*-epoxide **2** is geometrically matched for covalent reaction with double-stranded DNA to give the *trans*-adduct **3**. In contrast, the corresponding *endo*-epoxide fails to form an adduct at this or any site in DNA and is essentially nonmutagenic in contrast to the starkly mutagenic **2**.

In this paper we describe the isolation, characterization and attempted inactivation of vesicolorin B synthase (VBS) from *A. parasiticus*, the pivotal enzyme responsible for setting the absolute stereochemistry of the bisfuran ring system present in all members of the aflatoxin family. VBS catalyzes the cyclization of racemic versiconal hemiacetal (**8**) to optically active versicolorin B (**9**, Scheme 1). Interestingly, it was observed previously in cell-free experiments that *both* enantiomers of **8** reacted to give **9** (McGuire et al., 1989). The availability of pure cyclase (VBS) has allowed the question of whether one or more enzymes are required for this process to be answered. Preliminary purifications of VBS have been reported (Lin & Anderson, 1992; Townsend et al., 1992), but we detail here a revised and significantly improved protocol to obtain homogeneous protein, and we present extensive kinetics experiments to fully examine this key biosynthetic transformation.

The biosynthesis of aflatoxin B_1 (**1**) is outlined in Scheme 1 and is complex. A six-carbon starter unit (heavy line in **4**), evidently generated by a specialized fatty acid synthase (Brobst & Townsend, 1993; Mahanti et al., 1996; Yu et al., 1995), is homologated by successive reaction with seven malonyl units, cyclized, and oxidized to afford norsolorinic acid (**4**), the first anthraquinone intermediate in the pathway (Lee et al., 1971). Redox changes along the side chain give the internal ketal averufin (**5**) having the (1′*S*)-configuration (Koreeda et al., 1985). Oxidative rearrangement of this optically active substrate gives hydroxyversicolorone (**6**) (Townsend et al., 1988), which is isolated as its racemate (Townsend et al., 1988). The benzylic 2′-center is adjacent to an aldehyde, which, although masked as the hemiacetal of the first furan ring in **6**, is stereochemically labile despite its probable initial formation as a single stereoisomer (Townsend et al., 1989). Baeyer Villiger-like oxidation yields versiconal acetate (**7**), which is also obtained as its racemate (Schroeder et al., 1974). An esterase has been detected in a cell-free system from *A. parasiticus* that is capable of the hydrolysis of **7** to versiconal hemiacetal (**8**) (McGuire et al., 1989), an enzyme known to be inhibited by the phosphorus-based insecticide Dichlorovos (Schroeder et al., 1974). Versicolorin B synthase (VBS) catalyzes the critical ring closure of this racemic substrate to (−)-versicolorin B (**9**). The Baeyer Villiger-like oxidation of **6** to **7** has been demonstrated by the incorporation of molecular oxygen (*) specifically into the terminal acetate as shown in Scheme 1 (McGuire & Townsend, 1993). Hydrolysis gives **8** labeled with heavy isotope (*) which survives VBS-catalyzed cyclization, with presumed loss of the hemiacetal as water, to label the second furan ring as depicted in **9** (McGuire & Townsend, 1993). This cyclization process is the intramolecular analog of saccharide bond formation (Sinnott, 1990) and has motivated the design of potential mechanism-based inhibitors of this reaction. As it acts penultimately to formation of the lethal dihydrobisfuran eventually present in aflatoxin B_1 (**1**), VBS would be an ideal target for inhibitor synthesis.

Oxidative desaturation of **9** has been demonstrated to complete formation of the dihydrobisfuran in (−)-versicolorin A (**10**) (Yabe et al., 1991). The post-bisfuran steps of the biosynthetic pathway are less well-understood than the foregoing in which the anthraquinone nucleus is cleaved, decarboxylated, and deoxygenated to a xanthone. Successive

methylations give sterigmatocystin (**11**, R = H) and *O*-methylsterigmatocystin (**11**, R = Me) (Bhatnagar & Cleveland, 1988; Cleveland et al., 1987; Keller et al., 1993; Yabe et al., 1989) prior to further aryl oxidations, cleavage, demethylation, cyclization, and decarboxylation (Chatterjee & Townsend, 1994; Watanabe & Townsend, 1996) to afford aflatoxin B_1 (**1**).

MATERIALS AND METHODS

Materials. High-performance liquid chromatography was performed with a Waters 625 liquid chromatograph equipped with a model 484 variable wavelength UV−visible detector (Milford, MA). Radioactivity was measured using a Beckman LS 5801 liquid scintillation counter (Fullerton, CA) employing 15 mL of Opti-Fluor scintillation cocktail (Packard; Meriden, CT). Optical rotations were determined on the Perkin Elmer 141 polarimeter (Norwalk, CT). Analytical thin-layer chromatography was performed on Analtech GHLF Uniplate glass plates coated with silica gel (0.25 mm) impregnated with a fluorescent indicator (Newark, DE). Flash column chromatography utilized Merck Silica Gel 60 (230−400 mesh; Gibbstown, NJ). Dialysis was performed using Spectra/Por 12 000−14 000 MW cutoff (10 mm, 0.32 mL/cm or 25 mm, 2 mL/cm) molecular porous membranes tubing (Spectrum Medicinal Industries, Inc.; Houston, TX). Polyacrylamide gel electrophoresis was performed on a Hoefer Scientific Instruments SE 400 vertical slab gel electrophoresis unit with the buffer system of Laemmli (1970). Gels consisted of 7.5% polyacrylamide cross-linked with 0.68% bisacrylamide and were poured to a thickness of 1.5 mm. Soluble protein was quantified against a bovine serum albumin standard using Bio-Rad Protein Assay reagent based on the Bradford dye-binding procedure (Bradford, 1976). Native molecular weights of proteins were estimated by gel filtration using Pharmacia Superose 6 (Uppsala, Sweden) and Supelco Progel TSK-G5000 PWXL (Bellefonte, CA) columns, equilibrated with 50 mM potassium phosphate (pH 7.5) containing 20% glycerol. Proteins were eluted at 0.25 mL/min. β-Amylase (MW 200 000), alcohol dehydrogenase (MW 150 000), bovine serum albumin (MW 66 000), carbonic anhydrase (MW 29 000), and cytochrome *C* (MW 12 400) were used to calibrate the columns prior to analysis. Amino acid analyses were performed at the Johns Hopkins School of Medicine Protein/Peptide/DNA Facility. KaleidaGraph 2.1 (Abelbeck Software; Reading, PA) was employed for curve-fitting analyses. All chemicals and reagents were purchased from Aldrich or Sigma Chemical Corporation (St. Louis, MO, or Milwaukee, WI, respectively) unless otherwise stated. Dichlorovos was obtained from Shell Chemical Company (Houston, TX). Tritiated sodium acetate (2−5 Ci/mmol) was purchased from Amersham International (Arlington Heights, IL). Combustion microanalyses were performed at Atlantic Microlab, Inc. (Norcross, GA).

General Procedures

Fungal Strains and Culture Conditions. The wild-type strain utilized was an aflatoxingenic isolate of *A. parasiticus*, ATCC 56775, designated as SU-1. Stock cultures were maintained on slants of 3.8% potato dextrose agar, 0.5% yeast extract, and 0.5% Bacto-Agar, all obtained from Difco (Detroit, MI). Subcultures from these slants were grown on Petri dishes of this medium for 5−7 days (28 °C) in the dark prior to use. Submerged liquid cultures were fermented in the minimum mineral medium of Adye and Mateles (AM; 1964). The replacement medium (RM) utilized in whole-cell studies was the nitrogen-free resting cell broth of Hsieh (Lin et al., 1973) containing designated amounts of glucose. Cultures were incubated in the dark at 28 °C on a gyrotory shaker operating at 175 rpm.

Incorporation of [^{3}H]Sodium Acetate into Versiconal Hemiacetal Acetate. A 1 L portion of AM media contained in a 4 L Erlenmeyer flask was inoculated with SU-1 (1×10^8 conidia). After 48 h of incubation on a rotary shaker (175 rpm) at 28 °C in the dark, the cultures were checked for aflatoxin production by thin-layer chromatography (70:15:15 chloroform:acetone:hexanes; $R_f = 0.51$). The mycelial pellets were collected on sterile cheesecloth and rinsed with 200 mL of RM (10 g of glucose/L). Of this, 10 g (wet weight) was resuspended in 100 mL of RM (10 g of glucose/L) contained in a 250 mL Erlenmeyer flask. To this was added 100 μL of a 1% Dichlorovos solution in acetone (v/v), and 50 mCi of [^{3}H]sodium acetate dissolved in 0.5 mL of water. The flask was then connected to a circulating closed-atmosphere apparatus (Moore et al., 1985; Townsend & Krol, 1988). The incubation flask was connected to the system and shaken at 26−28 °C in the dark at 300 rpm. An artificial atmosphere of 1:1 N_2:O_2 gas was maintained for 48 h. The culture became bright orange during this time. After filtration of the mycelia onto cheesecloth, the cells were steeped in acetone (25 mL) in a 100 mL beaker for 2 h and then filtered. The acetone extract was concentrated, and the culture medium was extracted three times with 50 mL portions of a 1:1 mixture of ethyl acetate and chloroform. The organic phases were combined and concentrated *in vacuo*. Water (50 mL) was added, and the resulting aqueous phase was extracted three times with 50 mL portions of the 1:1 EtOAc:$CHCl_3$.[1] The organic phases were pooled and concentrated to dryness. To the orange solid was added 10 mg of unlabeled versiconal acetate as a carrier. This mixture was dissolved in EtOAc and filtered through silica gel. Flash chromatography on silica gel (5g, 60:20:15 hexanes:acetone:EtOAc) yielded partially purified versiconal acetate after concentration *in vacuo*, which was used without characterization.

Preparation of [^{3}H]Versiconal Hemiacetal. To a stirred solution of the tritiated versiconal acetate (*ca*. 25 mg) obtained above in 20 mL of methanol was added a saturated methanolic solution of potassium hydroxide (1 mL). The dark purple solution was stirred at room temperature for 1 h. The reaction mixture was neutralized by the addition of 50 mL of a 1 M KH_2PO_4 solution (pH 4) and extracted three times with 50 mL portions of a 1:1 EtOAc:$CHCl_3$. The organic layers were combined and concentrated *in vacuo*. Flash column chromatography on silica gel (3% MeOH in $CHCl_3$ with no acetic acid) yielded 16 mg of versiconal hemiacetal following removal of solvent *in vacuo*. The specific activity was estimated to be 0.59 Ci/mol; the total

[1] Abbreviations: AIBN, azabisisobutyronitrile; DMAP, 4-(dimethylamino)pyridine; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DMSO, dimethyl sulfoxide; DIPEA, diisopropylethylamine; EDTA, ethylenediaminetetraacetic acid; EtOAc, ethyl acetate; HMPA, hexamethyl phosphoramide; MES, 2-[*N*-morpholino]ethanesulfonic acid; MeOH, methanol; MOPS, 3-[*N*-morpholino]propanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; TBAF, tetrabutylammonium fluoride; TBDMSCl, *tert*-butyldimethylsilyl chloride; THF, tetrahydrofuran; TrisHCl, Tris(hydroxymethyl)aminomethane hydrochloride.

activity was 27 μCi, giving a total incorporation rate from sodium acetate of 0.06%.

Radiochemical Assay. [^{3}H]Versiconal hemiacetal was added from an HMPA stock solution into 10 mM potassium phosphate (pH 6.5) with 20% glycerol and 1 mM PMSF ("assay buffer") to a final concentration of 1% HMPA. Incubations were carried out in 1.5 mL Eppendorf micro test tubes at 37 °C. Reactions were stopped by the addition of 1 mL of EHA [1:1 diethyl ether in hexanes solution with 0.05% acetic acid (v/v)]. The organic phase was removed and run into a disposable pipette packed with a small plug of cotton and 0.3 g of silica gel, pre-equilibrated with 1 mL of EHA. The pre-equilibrated silica gel column was placed in a 20 mL glass scintillation vial. The incubation solution was extracted a second time with 1 mL of organic solvent mixture, and again the organic phase was applied to the silica gel. Once the solvent had eluted through, the column was rinsed with 3 mL of EHA. Scintillation cocktail (15 mL) was added to the resultant organic eluent and analyzed for the presence of [^{3}H]versicolorin B. The efficiency of isolating the cyclized product from the substrate was calculated to be 60% in control experiments utilizing samples with less than 3 nmol of radioactive (±)-versicolorin B (versicolorin C) dissolved in aqueous buffer. The identity of versicolorin B as the isolated compound from these assay conditions was supported by reverse phase chromatography and comparison to authentic material (McGuire et al., 1989).

pK_a Determination of Versiconal Hemiacetal. Spectrophotometric calculation of the acid dissociation constant of versiconal hemiacetal was performed as described by Albert and Serjeant (1984). To 2 mL portions of the constant ionic strength buffer system (Ellis & Morrison, 1982), 50 mM MES, 50 mM MOPS, and 100 mM Tris-HCl with 20% glycerol, whose pH had been adjusted within the range 5.9−7.5, was added 50 μL of a 1 mg/mL dimethylformamide solution of versiconal hemiacetal. The absorbance of the samples was measured at $\lambda = 319$ nm at 37 °C and compared to the absorbances of the non-ionized ($A_{mol} = 0.454$; pH 2) and ionized ($A_{ion} = 1.43$; pH 10) species prepared in a similar fashion in 20% glycerol to obtain estimates of the ionization constant. This wavelength was selected for its maximal difference between the two forms of anthraquinone chromophore, after analysis of the versiconal hemiacetal solutions at the two pH extremes (250−650 nm). The average of the results yielded a pK_a value of 6.8 ± 0.1.

*Optical Rotation of Versicolorin B (**9**)*. Partially purified versicolorin B synthase (VBS) from the isoelectric focusing (IEF) step of the purification procedure outlined below was used to generate enantiomerically pure versicolorin B (**9**) from racemic versiconal hemiacetal (**8**). To 6 mL of VBS-active Rotofor fractions was added 660 μL of 5 M NaCl to adjust the salt concentration to 0.5 M. After 30 min on ice, 100 mL of potassium phosphate buffer [50 mM potassium phosphate (pH 7.2), 20% glycerol, 0.1 mM PMSF, 0.1 mM benzamidine, and 1 mM EDTA] was added containing 150 μM versiconal hemiacetal (0.5% HMPA). The enzyme mixture was incubated at 30 °C on a rotary shaker for 18 h. The reaction was quenched by adjusting the NaCl concentration to 5 M (29.02 g) and extracting the aqueous mixture three times with an equal volume of EtOAc. The EtOAc extracts were pooled, dried over anhydrous $MgSO_4$, and evaporated to dryness on a rotory evaporator. The dry product mixture was dissolved in 1.0 mL of DMSO. To this was added 9.0 mL of acetonitrile, and the solution was analyzed by HPLC using a Phenomenex C_{18} column (10 μm; 250 mm × 9.4 mm) to determine the extent of reaction (92%). Reverse phase HPLC was carried out under isocratic conditions (60:40 acetonitrile:0.1% aqueous trifluoroacetic acid) to purify the versicolorin B produced (27 min) from the unreacted versiconal hemiacetal (12 min). The optical rotation of 3.00 mg of **9** in 1.100 mL of dioxane was measured and determined to be −0.586° at 25 °C, $[\alpha]_D^{25} = -214°$ ($c = 0.00273$ g/mL in dioxane); lit $[\alpha]_D^{25} = -223°$ ($c = 0.0042$ g/mL in dioxane) (Hamasaki et al., 1967).

Purification of VBS

Preparation of Cell-Free Extract. All procedures were carried out at 0−4 °C. The extraction buffer was 2.5 mM potassium phosphate (pH 7.5) 20% glycerol, with protease inhibitors (5 mM 1,10-phenanthroline monohydrate, 6 mM benzamidine hydrochloride hydrate, and 0.3 mM PMSF). Two one-liter cultures of SU-1 mycelia (60−65 h; 90−110 g wet weight) were filtered on cheesecloth, washed with distilled water (1500 mL), and submerged in liquid nitrogen. The frozen cells were disrupted with a mortar and pestle and placed in a 350 mL polycarbonate chamber. To this was added 100 g of acid-washed glass powder (0.1−0.11 mm) with approximately 150 mL of extraction buffer to fill the chamber. The suspension was then blended in a homogenizer equipped with a Teflon blade and an ice-water jacket (Bead-Beater; Biospec, Bartlesville, OK). Disruption duty cycles of 1 min were alternated with 1 min intervals at rest to facilitate cooling, for a total of 10 min. The resultant homogenate was then centrifuged at 20 000*g* for 30 min. Fungal metabolites and small molecules were removed immediately by diafiltration of the cell-free extract against 10 times the volume of extraction buffer using an Amicon Tangential Flow CH_2 System equipped with a 30 000 molecular weight cutoff Spiral Wound Membrane Cartridge.

Preparative Scale Isoelectric Focusing. To 50 mL of cell-free extract was added 1.25 mL of ampholyte solution (Ampholine pH 3.5−10, Pharmacia LKB Biotechnology; Milwaukee, Wisconsin). The solution was placed in a preparative isoelectric focusing (IEF) chamber (Rotofor, Bio-Rad; Melville, NY). The IEF chamber was cooled by a circulation bath of 50% ethylene glycol in water (−10 °C) through the entire procedure. An electric potential was established at constant power (12 W) to initiate the isoelectric focusing. After 5 h at constant power, the 20 IEF fractions were collected. Through previous analyses the activity was found to reside between the anode and the fraction with the most precipitate (between pH 4.0 ± 0.1 and pH 5.0 ± 0.1). These fractions were pooled and mixed with a cold aqueous solution of 20% glycerol to a final volume of 50 mL. To the 50 mL of enzyme solution was added 1.25 mL of ampholyte solution (Ampholine pH 4−6, Pharmacia LKB Biotechnology). This sample was added to the IEF chamber, cooled, and refocused at constant power (12 W). After an additional 5 h, the 20 IEF fractions were collected and $^1/_5$ of the volume of 300 mM KH_2PO_4 (pH 7.5) with 20% glycerol and protease inhibitors (described above) was added to reach Rotofor fraction. The fractions were analyzed for VBS activity. The active fractions (p*I* 4.7 ± 0.1) were pooled and stored at −20 °C.

Mono-Q Anion Exchange Chromatography. Mono-Q RH5/5 FPLC anion exchange column (5 mm × 50 mm, Pharmacia LKB Biotechnology) was equilibrated at room

temperature with 50 mM potassium phosphate (pH 7.5), 1 mM PMSF, and 20% glycerol (0.5 mL/min). 1 mL of the active Rotofor sample was loaded, and the column was rinsed with *ca*. 100 mL of buffer, A 2 h linear gradient was then initiated from 0 to 300 mM sodium chloride, and the eluent was monitored at $\lambda = 280$ nm. Versicolorin B synthase eluted between 130 and 140 mM NaCl.

Characterization of Versicolorin B Synthase

Effect of Ionic Strength on Versicolorin B Synthase. To a 10 mM potassium phosphate solution (pH 7.5) with 20% glycerol was added sodium chloride to final concentrations of 0, 50, 100, 200, and 400 mM NaCl. These solutions were used in a standard assay of versicolorin B synthase. To 80 μL portions of each of these solutions, containing 100 μM [^{3}H]versiconal hemiacetal and 1% HMPA, 20 μL of a partially purified enzyme solution was added. The assay mixtures were incubated for 0, 10, and 20 min in triplicate at 37 °C. The results of the triplicate runs were averaged and calculated as μM versicolorin B formed per minute of incubation as a function of added salt.

pH Analysis of Versicolorin B Synthase. A pH−rate profile of VBS was performed using a constant ionic strength buffer system consisting of 50 mM sodium formate, 50 mM MES, 100 mM Tris-HCl with 20% glycerol ("FMT"; pH 3.0−9.0) was utilized for this study (Ellis & Morrison, 1982). The pH of the system was varied by 0.5 pH units. For each individual pH studied, 10 μL of a solution of 8.5 mM [^{3}H]-versiconal hemiacetal dissolved in HMPA was added to 1.0 mL of the appropriate constant ionic strength buffer to achieve an average final substrate concentration of 85 mM (1% HMPA). To 90 μL portions of these buffered [^{3}H]-versiconal hemiacetal solutions was added 10 μL of either buffer (control) or a purified enzyme solution (4.2 μg/mL). Incubations of these mixtures were run in triplicate for 0 and 10 min. The results of the triplicate runs were averaged and calculated as rates of versicolorin B produced from each incubation (μM/min). The pH-dependent rates of enzymatic conversion of versiconal hemiacetal were taken as the difference between the rates of versicolorin B formation in incubations with enzyme and the velocities of cyclization measured in the controls. Apparent pK_a's of the enzyme−substrate complex were estimated by fitting the proton concentration-dependent data by nonlinear regression to eq 3 (Cleland, 1979). In this way the dual value of the two, similar dissociation constants (pK_a) was calculated to be 3.7 $\pm$ 0.1, and the singular basic pK_a (pK_b) was calculated to be 7.3 $\pm$ 0.1 (k[E] = 0.80 $\pm$ 0.15).

A pH−stability profile of VBS was performed using the same buffer system as described above, over the same pH range. To 10 μL portions of 2× FMT, at the appropriate pH (3.0−9.0), was added 10 μL of purified versicolorin B synthase solution [5.0 μg/mL; 5 mM potassium phosphate (pH 6.0), 20% glycerol], and the resulting mixture was incubated at 37 °C for 15 min. The pH of the system was varied by 0.5 pH units throughout the entire pH range. A stock solution of 30 μM [^{3}H]versiconal hemiacetal (9.3×10^{12} DPM/mol) was prepared in 200 mM potassium phosphate (pH 6.5) containing 20% glycerol and 1% HMPA. To each of these enzyme preincubation mixtures was added 80 μL of buffered [^{3}H]versiconal hemiacetal solution (30 μM), and the resulting mixtures were incubated for 10 min at 37 °C. Each assay was done in triplicate, the results were averaged, and the rates of versicolorin B produced were calculated from each incubation. The pH−stability profile was prepared by plotting the rate of enzymic conversion of versiconal hemiacetal as a function of the corresponding pH for each reaction mixture.

Determination of K_m and V_{max} for Versicolorin B Synthase. From a solution of [^{3}H]versiconal hemiacetal dissolved in assay buffer (*ca*. 50 mM; 1% HMPA), 50% serial dilutions were performed to obtain eight different substrate concentrations. The concentrations of each were determined radiochemically (200 μL in triplicate) and found to be 46, 33, 17, 8.8, 4.6, 2.3, 1.2, and 0.64 μM. Portions of these solutions (500 μL) were incubated in triplicate for 5 min with 10 μL of either assay buffer as a control or a dilute, purified enzyme solution (3.5 μg/mL). The results of the triplicate runs were averaged and calculated as μM versicolorin B produced per minute from incubation with enzyme over that of the control. The apparent K_m and V_{max} for VBS were calculated employing nonlinear regression and found to be 2.4 $\pm$ 0.3 μM and 0.14 $\pm$ 0.01 μM/min, respectively; the k_{cat} for VBS is therefore *ca*. 5 s^{-1}.

Time Course Studies. In order to kinetically determine the rate of racemization of versiconal hemiacetal in aqueous, buffered systems, the rate expression for VBS was refined to include possible competitive inhibition by the unreactive enantiomer (S′) and the product (P; eq 17) where $K_{S'}$ and K_P are the competitive inhibition constants of the inactive enantiomer and the product, respectively. The enzyme initial rates from the apparent K_m experiment were fit to the modified rate expression eq 17 as described in the Appendix. The value of V_{max} which was calculated previously (0.014 μM/min) was used to obtain a new estimation of K_m (1.04 $\pm$ 0.04 μM), based upon a single enantiomer of versiconal hemiacetal acting as substrate. Competitive inhibition by the corresponding antipode (K_S) was determined to be 14.5 $\pm$ 6.2 μM.

Estimation of K_P. Serial dilutions were made from a 114 μM solution of versicolorin B in assay buffer (1% HMPA) to obtain versicolorin B solutions of 114, 23, 11, 2.3, 1.1, 0.23, and 0 μM. Incubation mixtures consisted of 240 μL of these versicolorin B solutions, 240 μL of a 3.9 μM buffered solution of [^{3}H]versiconal hemiacetal (assay buffer with 1% HMPA), and 20 μL of either assay buffer as a control or purified enzyme solution (3 μg/mL). Seven final product concentrations (0, 0.11, 0.55, 1.1, 5.5, 11, and 55 μM) were thus analyzed. These mixtures were incubated in duplicate for 10 min each. The results of the runs were averaged and calculated as fractional inhibition (i). The value of K_P was determined by analysis of the data by nonlinear regression within the context of eq 6, where v_P and v_0 are the initial velocities at a given [S] in the presence and absence of product, respectively (Segel, 1975). K_P was found to be 11 $\pm$ 2 μM using the following values: [S] and [S′] = 0.983 μM; K_m = 1.04 μM; $K_{S'}$ = 14.5 μM.

Time Course Study. To 40 mL of assay buffer containing 1% HMPA was added 10 μL from a solution of 8.5 mM [^{3}H]versiconal hemiacetal in HMPA. To 490 μL portions of the resultant 2.14 μM buffered [^{3}H]versiconal hemiacetal solution was added 10 μL of either assay buffer or dilute, purified versicolorin B synthase solution (2.6 μg/mL). These mixtures were incubated at 37 °C in triplicate for 0, 2.5, 5.0, 10, 15, 20, 30, 40, 50, 60, 70, and 80 min and assayed for product. The results of the triplicate runs were averaged and calculated as μM versicolorin B produced from incuba-

tion with enzyme over that of the control. An estimation of V_{max} for the enzyme concentration analyzed came from the initial velocity within the first 10 min of incubation (0.14 ± 0.02 μM/min), providing an apparent first-order rate constant (k_{enz}) of 0.13 ± 0.05 min^{-1}. From the amount of product formed for each time period studied, values for the remaining versiconal hemiacetal concentration, [S] + [S′], were obtained as a function of time. These values of remaining versiconal hemiacetal were taken as approximations for the term [S′] after 30 min of incubation. A first-order rate constant (k_{obs}) was calculated to be 0.0138 ± 0.002 min^{-1} for the processing of the unreactive enantiomer subsequent to racemization. Solving for k_{rac} as described in the Appendix yielded a racemization rate constant of 0.015 ± 0.005 min^{-1}.

Inhibition Studies. Radiochemical assays were performed as described above using purified versicolorin B synthase (3.0 μg/mL). Initial inhibition experiments with VBS were conducted using radiochemical assays containing either 2-hydroxyphenylacetic acid (2.0 mM), 2,5-dihydroxyphenylacetic acid (2.0 mM), emodin (**14**, 750 μM), or 2,3-dihydro-2,4-dihydroxy-3-benzofuran ethanol (**15**, 50 μM) to determine the relative rates of versicolorin B formation. K_i determination of 2,3-dihydro-2,4-dihydroxy-3-benzofuran ethanol (**15**) was carried out at varying concentrations of inhibitor (0, 100, and 200 μM) while versiconal hemiacetal (**8**) was varied from 1.25 to 30 μM (1.25, 2.50, 10.0, and 30.0 μM). The enzyme assays were carried out for 10 min at 37 °C. Kinetic data were test fitted to the appropriate equations for competitive, noncompetitive, and uncompetitive inhibition with the FORTRAN programs of Cleland (1979).

*Synthesis of Hemiacetal Diol **15***

*2,3-Dihydro-4-hydroxy-2-{[tris(1-methylethyl)silyl]oxy}-3-benzofuran Ethanol (**18**)*. The known ester (**17**, 0.0163 g, 0.413 mmol) (Graybill et al., 1989) was dissolved in dry THF (2.5 mL, 0.016 M), and lithium aluminum hydride (0.0035 g, 0.0909 mmol) was added. The reaction mixture was stirred for 40 min at room temperature under argon. Water was added slowly to quench the excess reductant, and the reaction mixture was washed with 5% $NaHCO_3$, dried over brine, and concentrated to provide the diol **18** (0.011 g, 75%), which was used without further purification. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.76 (s, 1H, OH), 6.97 (t, J = 8.0 Hz, 1H, H-6), 6.39 (d, J = 8.1 Hz, 1H, H-7), 6.32 (d, J = 7.9 Hz, 1H, H-5), 5.85 (s, 1H, H-2), 4.48 (s, 1H, OH), 3.78 (m, 1H, H-2′), 3.67 (m, 1H, H-2′), 3.36 (t, J = 7.4 Hz, 1H, H-3a), 1.93 (m, 1H, H-1′), 1.77 (m, 1H, H-1′), 1.05−1.14 (m, 21H, TIPS); ^{13}C NMR (300 MHz, acetone-d_6) δ 160.6 (s, 1, C-7a), 155.6 (s, 1, C-4), 129.7 (s, 1, C-6), 115.8 (s, 1, C-3a), 109.6 (s, 1, C-2), 107.3 (s, 1, C-5), 102.3 (s, 1, C-7), 60.6 (s, 1H, H-2′), 47.8 (s, 1, C-3), 35.2 (s, 1, H-1′), 18.2 (s, 1, Si*CH*CH_3), 18.1 (s, 1, Si*CH*CH_3), 12.9 (s, 1, SiCH*CH*$_3$); IR ($CHCl_3$) 3391, 2946, 2866, 2254, 1696, 1465, 1250, 1030 cm^{-1}; MS (EI) calcd for $C_{19}H_{32}O_4Si$, *m/e* (relative intensity) 352.2070, found 352.2066; 352 (M^+, 1), 309 (17), 291 (18), 279 (13), 178 (100), 161 (24), 150 (14), 132 (27), 103 (33), 75 (29), 61 (18).

*2,3-Dihydro-2,4-dihydroxy-3-benzofuran Ethanol (**15**)*. The TIPS acetal (**18**, 0.0640 g, 0.182 mmol) was dissolved in dry THF (3.6 mL, 0.05 M), and TBAF (95% pure) was added as a 1 M solution in THF (0.210 mL, 0.200 mmol). The yellow-orange solution was stirred at room temperature for 30 min under argon and directly purified by radial chromatography (Chromatotron, 50% EtOAc/hexanes) to provide the desired triol as white needles (0.0314 g, 88%; mp 132.0−134.0 °C). ^{1}H NMR (300 MHz, acetone-d_6) δ 6.93 (t, J = 8.1 Hz, 1H, H-6), 6.37 (d, J = 8.1 Hz, 1H, H-7), 6.23 (d, J = 7.4 Hz, 1H, H-5), 6.23 (d, J = 5.9 Hz, 1H, H-2), 3.78 (t, J = 7.1 Hz, 1H, H-3), 3.98 (t, J = 7.4 Hz, 1H, H-2′), 3.46 (m, 1H, H-2′), 2.17 (d, J = 6.1 Hz, 1H, H-1′), 2.12 (m, 1H, H-1′), 1.05−1.14 (m, 21H, TIPS); ^{13}C NMR (300 MHz, acetone-d_6) δ 162.1 (s, 1, C-7a), 155.3 (s, 1, C-4), 130.2 (s, 1, C-6), 114.2 (s, 1, C-3a), 112.1 (s, 1, C-2), 109.0 (s, 1, C-5), 101.2 (s, 1, C-7), 67.6 (s, 1H, H-2′), 45.5 (s, 1, C-3), 32.2 (s, 1, H-1′); IR ($CHCl_3$) 3610, 3375, 2960, 2932, 2875, 2856, 1635, 1616, 1465, 1187, 1078 cm^{-1}; MS (EI) calcd for $C_{10}H_{10}O_3$, *m/e* (relative intensity) 178.0630; found 178.0627 (M − H_2O); 178 (100), 163 (44), 149 (23), 135 (10), 121 (12), 103 (13), 91 (9), 77 (15), 65 (10), 57 (9), 51 (9), 43 (28).

*Synthesis of Styrene Oxide **16***

*Methyl [4-Chloro-3-(2′-propenyloxy)]benzoate (**23**)*. 4-Chloro-3-hydroxybenzoic acid (**22**, 9.757 g, 56.53 mmol) was dissolved in reagent-grade methanol (220 mL, 0.25 M), and concentrated H_2SO_4 (5.546 g, 56.53 mmol) was added dropwise, causing the reaction color to darken to a rusty brown. The reaction mixture was stirred and heated to reflux for 2.5 h under an argon atmosphere. The bulk of the methanol was removed under reduced pressure, and the remainder of the reaction mixture was partitioned between H_2O and EtOAc. The aqueous phase was extracted (4×) with EtOAc, and the extracts were pooled, washed with 2 N NaOH, saturated brine, and dried over anhydrous $MgSO_4$. Silica gel chromatography eluting with a 5−10% ethyl acetate/hexanes gradient yielded the methyl ester as a white solid (8.41 g, 80%; mp 97.0−98.0 °C).

The ester (1.965 g, 10.53 mmol) was dissolved in reagent-grade acetone (105 mL, 0.1 M) and treated with K_2CO_3 (1.601 g, 11.58 mmol) and allyl bromide (1.209 g, 15.80 mmol). The reaction mixture was heated to reflux and stirred overnight under nitrogen, the color fading from orange to pale yellow. The reaction mixture was poured into water and the aqueous phase was extracted with EtOAc (4×). The organic extracts were pooled, washed with 2 N NaOH (2×), 5% $NaHCO_3$, and brine, and dried over anhydrous $MgSO_4$. The solvent was removed under reduced pressure, and the residue was purified by silica gel chromatography (column: 4 cm × 16 cm, 5% ethyl acetate/hexanes) to yield the ether **23** as a white solid (2.243 g, 94%; mp 41.5−42.5 °C). ^{1}H NMR (300 MHz, CD_2Cl_2) δ 7.42 (dd, J = 8.5, 1.8 Hz, 1H, H-6), 7.42 (d, J = 1.8 Hz, 1H, H-2), 7.28 (d, J = 8.7 Hz, 1H, H-5), 6.01 (ddt, J = 17.2, 10.7, 5.0 Hz, 1H, H-2′), 5.40 (ddd, J = 17.2, 3.2, 1.4 Hz, 1H, H-3′*E*), 5.22 (ddd, J = 10.6, 2.9, 1.1 Hz, 1H, H-3′*Z*), 4.53 (ddd, J = 5.0, 1.4, 1.1 Hz, 2H, H-1′), 3.79 (s, 3H, OCH_3); IR ($CHCl_3$) 3026, 2954, 1720, 1581, 1486, 1439, 1414, 1294, 1241, 1108, 1060, 1019, 934, 876 cm^{-1}. Anal. Calcd for $C_{11}H_{11}O_3Cl$: C, 58.29%; H, 4.89%; Cl, 15.64%. Found: C, 58.22%; H, 4.91%; Cl, 15.74%.

*Methyl 2-Allyl-4-chloro-3-hydroxybenzoate (**24**)*. The allyl ether **23** (0.1946 g, 0.8622 mmol) was dissolved in reagent-grade chloroform (3.5 mL, 0.25 M), and diethylaluminum chloride was added as a 1.0 M solution in heptanes (2.6 mL, 2.6 mmol). The reaction mixture was heated to reflux for

25 min under a nitrogen atmosphere, and the resulting yellow solution was poured into a separatory funnel containing ice cold 5% HCl. The aqueous phase was extracted (3×) with chloroform, and the organic extracts were pooled, washed with 5% $NaHCO_3$ and brine, and dried over anhydrous $MgSO_4$. The solvent was removed under reduced pressure leaving 0.1670 g of the phenol **24** as a white solid (86%; mp 86.0−86.5 °C). Chromatography was not required. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.38 (d, J = 8.5 Hz, 1H, H-6), 7.23 (d, J = 8.5 Hz, 1H, H-5), 5.99 (ddd, J = 17.1, 10.2, 6.2 Hz, 1H, H-2′), 5.04 (ddd, J = 10.4, 3.3, 1.7 Hz, 1H, H-3′*E*), 5.00 (ddd, J = 17.1, 3.4, 1.7 Hz, 1H, H-3′*Z*), 3.86 (s, 3H, CH_3), 3.79 (dt, J = 6.2, 1.5 Hz, 2H, H-1′); IR ($CHCl_3$) 3536, 3029, 3009, 2958, 1723, 1595, 1436, 1262, 1145, 920 cm^{-1}. Anal. Calcd for $C_{11}H_{11}O_3Cl$: C, 58.29%; H, 4.89%; Cl, 15.64%. Found: C, 58.21%; H, 4.88%; Cl, 15.70%.

*Methyl 4-Chloro-3-(tert-butyldimethylsilyloxy)-2-(3′-bromoprop-1′E-enyl)benzoate (**25**).* The phenol **24** (0.3212 g, 1.461 mmol) was dissolved in dry THF (15 mL, 0.1 M), and TBDMSCl (0.5527 g, 3.668 mmol) and DIPEA were added (0.3795 g, 2.935 mmol). The mixture was stirred for 8 h at room temperature under argon, whereupon a second equivalent addition of both reagents was made and the solution was stirred a further 24 h. The reaction mixture was poured into 2 N NaOH, and the aqueous phase was extracted (3×) with ethyl acetate. The extracts were pooled, washed with brine, and dried over anhydrous $MgSO_4$. The solvent was removed under reduced pressure, leaving 0.4888 g of the silyl ether as a light yellow oil (98%). Chromatography was not necessary.

The protected olefin (0.298 g, 0.874 mmol) was dissolved in reagent-grade carbon tetrachloride (8.7 mL, 0.1 M), and *N*-bromosuccinimide (0.233 g, 1.31 mmol) and AIBN (0.646 g, 0.393 mmol; recrystallized from ethanol) were added resulting in a white slurry. The mixture was heated to reflux and stirred under argon for 6 h and then placed directly on a silica gel column. Open column silica gel chromatography using pentane as eluent provided the bromide **25** as a colorless oil (0.260 g, 71%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.31 (d, J = 8.4 Hz, 1H, H-6), 7.28 (d, J = 8.4 Hz, 1H, H-5), 6.87 (dt, J = 15.8, 1.1 Hz, 1H, H-1′), 5.99 (dt, J = 15.8, 7.6 Hz, 1H, H-2′), 4.10 (dd, J = 7.6, 1.1 Hz, 1H, H-3′), 3.87 (s, 3H, COOMe), 1.02 (s, 9H, Si-*t*-Bu), 0.18 (s, 6H, SiMe); IR ($CHCl_3$) 2952, 2935, 2860, 1722, 1576, 1472, 1464, 1435, 1414, 1285, 1256, 1152, 1131, 1014, 969, 935, 835 cm^{-1}. Anal. Calcd for $C_{17}H_{24}O_3SiClBr$: C, 48.64%; H, 5.76%; Br, 19.03%; Cl, 8.44%. Found: C, 48.75%; H, 5.81%; Br, 19.08%; Cl, 8.47%.

*Methyl 4-Chloro-3-(tert-butyldimethylsilyloxy)-2-(3′-hydroxyprop-1′E-enyl)benzoate (**26**).* Bromide **25** (0.7400 g, 1.762 mmol) in a three-necked round-bottomed flask was dissolved in thiophene-free benzene (*ca*. 20 mL, *ca*. 0.1 M). The flask was equipped with a stir bag, gas outlet, septum, and a thermometer adapter which held a gas dispersion tube. In-house compressed air was passed through a $CaSO_4$ drying tower and bubbled into the solution for 15 min. Slow, dropwise addition of Bu_3SnH (1.060 g, 4.410 mmol) was carried out over 5 min. The reaction mixture was stirred at room temperature for 46 h with bubbling of dry air. At 12 h another equivalent addition of Bu_3SnH was made. The resulting orange solution was concentrated under an increased flow of dry air. The residue was brought up in "wet ether" and treated with 6 drops of DBU to remove the tin salts as a yellow precipitate (Bu' Lock, 1974). The reaction mixture was loaded directly onto a silcia gel column and eluted (0−20% EtOAc/hexanes gradient) providing the allylic alcohol **26** as a light yellow oil (0.4016 g, 65%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.31 (d, J = 8.4 Hz, 1H, H-6), 7.27 (d, J = 8.4 Hz, 1H, H-5), 6.81 (dt, J = 16.2, 1.6 Hz, 1H, H-1′), 5.98 (dt, J = 16.2, 5.3 Hz, 1H, H-2′), 4.30 (dd, J = 5.3, 1.6 Hz, 1H, H-3′), 3.84 (s, 3H, COOMe), 1.01 (s, 9H, *t*-Bu), 0.17 (s, 6H, Si-Me_2); IR ($CHCl_3$) 3615, 3502, 2952, 2933, 2859, 1718, 1434, 1416, 1286, 1258, 1155, 1021, 932, 839 cm^{-1}. Anal. Calcd for $C_{17}H_{25}O_4SiCl$: C, 57.21%; H, 7.06%; Cl, 9.93%. Found: C, 57.06%; H, 7.12%; Cl, 10.08%.

*4-Chloro-3-(tert-butyldimethylsilyloxy)-2-(3′-tert-butyldimethylsilyloxyprop-1′E-enyl)benzyl Alcohol (**27**).* The alcohol **26** (0.4770 g, 1.337 mmol) was dissolved in dry dichloromethane (13.4 mL, 0.1 M), and TBDMSCl (0.3021 g, 2.005 mmol), DMAP (0.0164 g, 0.1337 mmol), and reagent-grade triethylamine (0.2029 g, 2.005 mmol) were added. The reaction mixture was stirred at room temperature for 20 h under argon, and the mixture was partitioned between dichloromethane and water. The organic extracts were pooled, washed with cold 5% HCl, 1 N NaOH, and brine, and dried over anhydrous $MgSO_4$. The solution was concentrated under reduced pressure and the product was purified by radial chromatography [Chromatotron, 2 mm rotor, 5−10% EtOAc/hexanes gradient] to furnish the analytically pure (data not given) bissilyl ether as a colorless oil (0.4045 g, 84%).

The bissilyl ether (0.2813 g, 0.5970 mmol) was dissolved in dry ether (12.0 mL, 0.05 M), and DIBAL was added (1.25 mL, 1.254 mmol) as a 1.0 M solution in hexanes at a rapid rate. The solution was stirred at room temperature under argon for 30 min and poured into a separatory funnel. Water was added cautiously, and the resulting phases were separated. The aqueous layer was extracted with ethyl acetate (3×), and the organic extracts were pooled and dried with brine and anhydrous $MgSO_4$ and finally filtered through a plug of silica gel. The benzyl alcohol **27** was obtained as a yellow oil (0.2449 g, 93%), which was carried on directly to the next step. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.26 (d, J = 8.3 Hz, 1H, H-6), 7.06 (d, J = 8.3 Hz, 1H, H-5), 6.64 (dt, J = 16.2, 1.9 Hz, 1H, H-1′), 6.08 (dt, J = 16.2, 4.5 Hz, 1H H-2′), 4.65 (s, 2H, Bn), 4.39 (dd, J = 4.5, 1.9 Hz, 1H, H-3′), 2.62 (br s, 1H, OH), 1.07 (s, 9H, *t*-Bu), 0.99 (s, 9H, *t*-Bu), 0.23 (s, 6H, Me), 0.16 (s, 6H, Me); ^{13}C NMR (300 MHz, $CDCl_3$) δ 149.2 (s, 1, C-3), 138.4 (s, 1, C-1), 135.6 (s, 1, C-1′), 130.5 (s, 1, C-2), 128.3 (s, 1, C-5), 125.0 (s, 1, C-4), 123.0 (s, 1, C-2′), 121.8 (s, 1, C-6), 63.5 (s, 1, C-3′), 62.9 (s, 1, Bn), 26.0 (s, 3, CMe_3), 25.9 (s, 3, CMe_3), 18.6 (s, 1, CH), 18.3 (s, 1, CH), −3.0 (s, 2, $SiMe_2$), −5.4 (s, 2, $CiMe_2$); IR ($CHCl_3$) 3607, 2931, 2860, 1473, 1463, 1423, 1256, 1129, 988, 841 cm^{-1}; MS (EI) calcd for $C_{22}H_{39}O_3Si_2Cl$, *m*/*e* (relative intensity) 385.1422; found 385.1428; 398 (9), 385 (M^+, 22), 255 (11), 253 (30), 225 (15), 223 (11), 189 (12), 179 (27), 147 (82), 105 (68), 91 (31), 73 (100).

*4-Chloro-3-(tert-butyldimethylsilyloxy)-2-(3′-tert-butyldimethylsilyloxyprop-1′E-enyl)benzaldehyde (**28**).* The benzoyl alcohol **27** (0.2073 g, 0.4467 mmol) was dissolved in dry dichloromethane (4.68 mL, 0.1 M) and treated with Dess−Martin periodinane (Dess & Martin, 1983). The reaction mixture was stirred at room temperature under argon for 45 min, whereupon the mixture was placed directly on a silica

gel column and eluted (10% EtOAc/hexanes) to yield aldehyde **28** (0.1816 g, 88%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.08 (s, 1H, CHO), 7.48 (d, J = 8.4 Hz, 1H, H-5), 7.36 (d, J = 8.4 Hz, 1H, H-4), 6.87 (d, J = 15.8 Hz, 1H, H-1′), 5.80 (dt, J = 15.8, 3.9 Hz, 1H, H-2′), 4.40 (dd, J = 3.9, 2.2 Hz, 2H, H-3′), 1.03 (s, 9H, *t*-Bu), 0.94 (s, 9H, *t*-Bu), 0.22 (s, 6H, Me), 0.11 (s, 6H, Me); IR ($CHCl_3$) 2958, 2933, 2858, 1685, 1573, 1472, 1423, 1259, 1132, 1013, 913, 839, 749 cm^{-1}. Anal. Calcd for $C_{22}H_{37}O_3Si_2Cl$: C, 59.90%; H, 8.45%; Cl, 8.04%. Found: C, 60.12%; H, 8.17%; Cl, 8.06%.

*4-Chloro-3-hydroxy-2-(3′-hydroxyprop-1′E-enyl)benzaldehyde (**29**).* The aldehyde **28** (0.1337 g, 0.3030 mmol) was dissolved in a 10:1 DMSO/H_2O solution (1.2 mL, 0.25 M) and treated with NBS (0.1672 g, 0.3994 mmol). The resulting mixture was stirred at room temperature for 48 h before being poured into water and ether. The aqueous phase was partitioned and extracted (2×) with EtOAc. The extracts were pooled, washed with water (3×) and brine, and dried over anhydrous $MgSO_4$. The residue was separated by radial chromatography (Chromatotron, 1 mm rotor, 30% EtOAc/hexanes) to furnish the aldehyde **29** as a white solid (0.0382 g, 59%; mp 69.5−71.5 °C). ^{1}H NMR (300 MHz, acetone-d_6) δ 10.14 (s, 1H, CHO), 7.43 (d, J = 8.5 Hz, 1H, H-6), 7.37 (s, J = 8.5 Hz, 1H, H-5), 6.99 (dt, J = 16.0, 1.8 Hz, 1H, H-1′), 6.09 (dt, J = 16.0, 4.7 Hz, 1H, H-2′), 4.35 (dd, J = 4.7, 1.8 Hz, 2H, H-3′); ^{13}C NMR (300 MHz, acetone-d_6) δ 191.9 (s, 1, CHO), 151.5 (s, 1, C-3), 142.4 (s, 1, C-1′), 134.9 (s, 1, C-1), 131.6 (s, 1, C-2), 128.9 (s, 1, C-5), 126.4 (s, 1, C-4), 120.9 (s, 1, C-2′), 120.3 (s, 1, C-6), 62.9 (s, 1, C-3′); IR (CH_3Cl) 3528, 3409, 3235, 3034, 1779, 1756, 1715, 1541, 1431, 1343, 1312, 1293, 1239, 1179, 1161, 1147, 941, 757 cm^{-1}; MS (CI, NH_3) calcd for $C_{10}H_9O_3Cl$, *m/e* (relative intensity) 230.0584; found 230.0586 (M + NH_4); 214 (0.3), 212 (M^+, 0.7), 197 (0.9), 195 (3.0), 183 (32), 181 (100), 165 (8).

*4-Chloro-3-hydroxy-2-(3′-hydroxyprop-1′E-enyl)styrene Oxide (**16**).* Trimethylsulfonium iodide (0.120 g, 0.588 mmol) was dissolved in reagent-grade DMSO (4.0 mL, 0.05 M), and 95% NaH (0.0146 g, 0.608 mmol) was added. The reaction mixture was stirred for 1.5 h at room temperature under argon, and a DMSO solution (12 mL, 0.05 M) of the aldehyde **29** (0.0417 g, 0.1961 mmol) was cannulated into the flask containing the sulfur ylide in a dropwise manner. The resulting mixture was stirred for 21 h at room temperature and then poured into EtOAc and water. The organic phase was washed with water (3×) and brine and dried over anhydrous $MgSO_4$. Purification by radial chromatography (Chromatotron, loaded and run with 50% EtOAc/hexanes) provided epoxide **16** (0.0049 g, 11%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.99 (s, 1H, OH), 7.21 (d, J = 8.5 Hz, 1H, H-6), 6.83 (d, J = 8.2 Hz, 1H, H-5), 6.79 (dt, J = 16.1, 1.6 Hz, 1H, H-1′), 6.39 (dt, J = 16.2, 5.3 Hz, 1H, H-2′), 5.95 (br s, 1H, OH), 4.40 (dd, J = 5.1, 1.1 Hz, 2H, H-3′), 4.03 (dd, J = 4.0, 2.7 Hz, 1H, Bn), 3.16 (dd, J = 5.7, 4.1 Hz, 1H, epx), 2.70 (dd, J = 5.7, 2.6 Hz, 1H, epx); ^{13}C NMR (400 MHz, CD_3Cl) δ 149.3 (s, 1, C-3), 136.4 (s, 1, C-1′), 135.8 (2, 1, C-1), 127.6 (s, 1, C-5), 124.4 (s, 1, C-2), 122.3 (s, 1, C-2′), 119.6 (s, 1, C-4), 117.1 (s, 1, C-6), 63.8 (s, 1, C-3′), 50.8 (s, 1, epx), 50.6 (s, 1, epx); IR ($CDCl_3$) 3506, 3402, 2872, 1645, 1470, 1304, 1281, 1229, 1134, 1096, 1030 cm^{-1}; MS (CI, NH_3) calcd for $C_{11}H_{12}O_3Cl$, *m/e* (relative intensity) 227.0475, found 227.0475 (M + H); 211 (36), 209 (100), 197 (6), 195 (11), 171 (34).

RESULTS AND DISCUSSION

Radiochemical Assay. Despite the capability of reverse phase HPLC to quantify tetrahydrobisfuran production in enzymatic incubations (Townsend et al., 1992), the sensitivity of this method was found to be inadequate to detect low extents of turnover with the accuracy necessary to carry out reproducible kinetic studies. A more sensitive technique was developed to accurately monitor the appearance of versicolorin B. The ability to incorporate acetate into this metabolite was exploited as an efficient means to obtain radiolabeled substrate. Wild-type mycelia at the onset of aflatoxin production were incubated with [^{3}H]acetate (50 mCi, 2−5 Ci/mmol) in the presence of Dichlorovos. After 48 h of incubation, the mycelia and medium were separately extracted and the resultant material was combined with unlabeled versiconal hemiacetal acetate as a carrier. Partial purification by flash chromatography and saponification of the radiolabeled versiconal acetate afforded, following silica gel chromatography, [^{3}H]versiconal hemiacetal with a specific activity of 0.6 Ci/mol. The total incorporation rate of radioisotope was found to be 0.06%, consistent with previously reported incorporation experiments (Singh & Hsieh, 1977; Steyn et al., 1979).

An efficient method of separating the substrate and product from the versicolorin B synthase enzyme assay mixture was developed using a 1:1 mixture of diethyl ether and hexanes with 0.05% acetic acid (v/v) to elute versicolorin B while the considerably more polar unreacted versiconal hemiacetal substrate remained bound to the silica gel. As long as the proper controls were simultaneously run, the amount of biosynthetically derived product could be distinguished from that resulting from chemical cyclization. Replicate runs were employed to obtain averaged data.

Once the protocol for separation and quantification of versicolorin B had been developed, the conditions of the assay were analyzed in order to maximize enzyme activity. A highly polar solvent was necessary to first dissolve the versiconal hemiacetal and then to maintain its solubility in aqueous buffer. Analysis of a variety of organic solvents indicated that hexamethylformamide (HMPA), at final concentrations of less than 2%, exhibited negligible effects on VBS activity, unlike other aprotic, polar solvents such as dimethyl sulfoxide and dimethylformamide, which were found to significantly destabilize VBS. Based on initial VBS pH−rate profiles, employing a constant ionic strength buffer (Ellis & Morrison, 1982), a value of pH 6.5 was chosen to optimize its conversion to versicolorin B in conjunction with low extents of background chemical cyclization.

Isolation of VBS. Prior reports of the purification of versicolorin B synthase (Lin & Anderson, 1992; Townsend et al., 1992) have described a variety of chromatographic procedures to obtain the desired protein from cell-free extracts of *A. parasiticus*. Anion exchange (DEAE-Sepharose, DEAE-Cellulose, Mono-Q), hydrophobic interaction (ethyl agarose, phenyl-RP$^+$), hydroxyapatite and dye−ligand (Reactive Green 19 and Red 120 agarose, Matrix Green A) chromatographic resins have all been shown to give useful purifications of the protein. Several combinations of these techniques, however, have failed in our hands to provide a reproducible means to homogeneous VBS and have produced only minute amounts of protein. Furthermore, in previously reported purification schemes ammonium sulfate precipitation was employed as an initial step; a drastic

Table 1: Purification of VBS from *A. parasiticus*

purification step	protein (μg/mL)	protein (mg)	total activity (μM/min)	specific activity (μM/min·mg)	yield (%)	purification (*n*-fold)
cell-free extract	1600	65	2900	36	100	1
Rotofor IEF	220	1.6	580	360	20	10
Mono-Q	10	0.085	160	1900	5.5	53

reduction in overall activity (<2%) was observed in these laboratories upon protein precipitation with salt. The specific activity of the resuspended, exhaustively dialyzed ammonium sulfate pellet was also greatly diminished (<4% of the original cell-free extract), a phenomenon which was only partially reversed in subsequent chromatographic steps with concomitant removal of an orange pigment that co-precipitated with the protein. It is unclear whether this component of the ammonium sulfate pellet is responsible for inhibition of the enzyme, or if the change in hydropathy caused by the high salt concentration is affecting another aspect of the protein structure.

Both the cumbersome number of chromatographic steps and the very large losses of activity in the orange ammonium sulfate precipitate were circumvented, however, by exploiting the low isoelectric point of VBS. Employing preparative-scale isoelectric focusing technology (Rotofor; Bio-Rad), versicolorin B synthase can be efficiently separated from the majority of contaminating proteins. Homogenization of the mycelia in low salt buffer (2.5 mM potassium phosphate with 20% glycerol; pH 7.5) was followed, after centrifugation, by the addition of an ampholite solution capable of buffering in the range pH 3.5−10. In an electric potential the majority of the proteins migrate and then precipitate at pH 5. This method is also advantageous in that it effectively removed nucleic acids, which accumulated near the anode. Experiments performed on partially-purified enzyme indicated that VBS possesses a p*I* value of 4.7 ± 0.1. pH−activity relationship studies (see below) revealed that the enzyme is active and stable at this low pH. The separation of VBS from contaminating proteins was further enhanced by an additional isoelectric focus, wherein the active fractions from above were pooled, and brought up to 60 mL with a 20% glycerol solution. Ampholine (pH 4−6) was added to the IEF pool to a final concentration of 2% (v/v), and a new potential applied to expand the narrower pH range (4.0−6.0) across the chamber. This double-focusing method afforded partially-purified protein with a 10-fold increase in specific activity. While the total recovered activity was *ca*. 70%, only the purest fractions were combined and carried on to the next step (*ca*. 20%).

Homogeneous versicolorin B synthase was afforded after FPLC anion exchange chromatography (Mono-Q HR 5/5, Pharmacia). After the column was equilibrated in 50 mM potassium phosphate with 20% glycerol (pH 7.5), a linear gradient from 0 to 300 mM NaCl gave the active protein at a chloride concentration between 130 and 140 mM. Although recovery is low for this step (<30%), the 5-fold purification yielded homogeneous protein. Thus, in two steps, avoiding the detrimental ammonium sulfate fractionation procedure, pure VBS could be isolated in active form for physical and mechanistic characterization in 5−6% overall yield (Table 1; Figure 1).

Physical Characterization. The molecular weight of the native protein was estimated by use of a calibrated HPLC-grade gel filtration column, which provided a value of

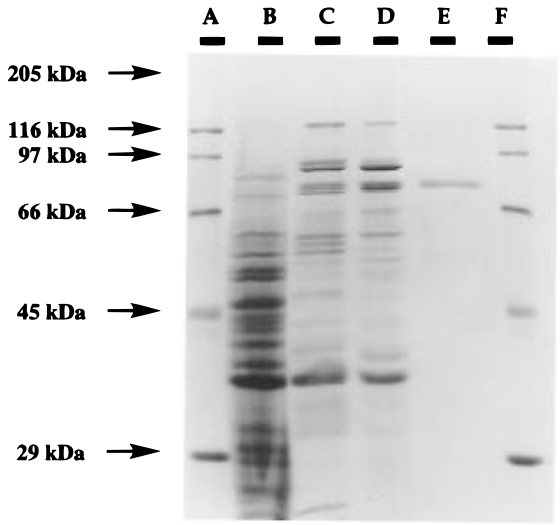


FIGURE 1: SDS−polyacrylamide gel electrophoresis monitoring the progress of the isolation of versicolorin B synthase. Lane A, molecular weight markers as shown; lane B, cell-free extract, lane C, Rotofor IEF focus 1 (pH 3.5−10); lane D, Rotofor IEF focus 2 (pH 4−6); lane E, Mono-Q anion exchange chromatography; lane F, molecular weight markers as shown.

Table 2: Amino Acid Analysis of Versicolorin B Synthase

amino acid	content (mol %)	amino acid	content (mol %)
Asx	7.44	Tyr	2.66
Glx	9.16	Val	5.08
Ser	9.30	Met	1.99
Gly	15.30	Cys	0.32
His	1.54	Ile	3.71
Arg	5.05	Leu	8.42
Thr	6.17	Trp	ND[a]
Ala	9.62	Phe	3.93
Pro	6.96	Lys	3.36

[a] ND, not determined.

140 000 ± 15 000 Da. This result in conjunction with the molecular weight as determined by SDS gel electrophoretic mobility (78 000 ± 2500 Da) is consistent with a dimer of identical subunits.

While partially purified protein was yellow in color and gave a UV spectrum vaguely suggestive of flavin binding, the concentration of this pigment was 5−10 times too low in proportion to the molar amount of the enzyme. In contrast, the homogeneous enzyme obtained in the present purification scheme gave no indication of a chromophoric cofactor and only typical absorption at 280 nm.

Amino acid analysis was performed on isolated versicolorin B synthase after elution through a Superose 6 gel filtration FPLC column equilibrated in 10 mM potassium phosphate (pH 6.5) containing no glycerol. The protein was then examined on a Waters PICO-TAG amino acid analysis system after acid hydrolysis (Perham, 1978). The results appear in Table 2.

The ionic strength of a solution indicates the bulk ionic environment to which a protein is subjected. Changes in the environment can modify charged interactions and effect

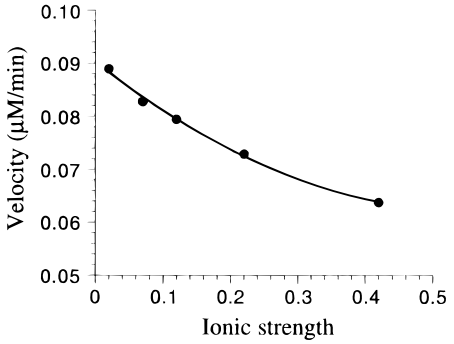


FIGURE 2: Effect of ionic strength on versicolorin B synthase activity.

protein structure and activity. The ionic strength of a series of VBS incubations was varied by the addition of solid sodium chloride to 10 mM potassium phosphate (pH 7.5, $I = 0.022$) containing 20% glycerol; the results appear in Figure 2. The data indicate an inverse relationship between ionic strength and VBS activity. This effect is consistent with either a highly ionic active site less capable of catalyzing acetal formation due to increases in the ionic environment, a nonpolar binding site which has an increased affinity for the more hydrophobic versicolorin B, or a change in tertiary structure of the protein. Regardless, the effect is significant enough to maintain a low ionic strength in order to maximize the conversion of versiconal hemiacetal. A concentration of 10 mM potassium phosphate was selected, therefore, as a means to sustain both pH and low ionic strength for each assay.

In order to identify ionizations that effect the catalytic activity of VBS and thereby discover the possible identities of any catalytic amino acid residues, pH−rate and pH−stability profiles were performed with homogeneous protein. The changes in activity observed under varying pH conditions are directly related to ionizations occurring within the active site of the enzyme−substrate complex. In the present experiments a constant, large quantity of substrate was used at every pH increment to approximate V_{max} conditions (Fersht, 1985; Tipton & Dixon, 1979). A combination of 50 mM sodium formate, 50 mM MES, and 100 mM Tris-HCl with 20% glycerol was chosen due to the large separation of the pK_a values of the individual components which act to provide a near-constant ionic strength of 0.1 over a pH range 3.0−9.0 (Ellis & Morrison, 1982). The pH of this buffer was adjusted by increments of 0.5 unit, and triplicate activity assays were performed of purified VBS in the presence of 80 μM [^{3}H]versiconal hemiacetal for each pH studied. The results appear in Figure 3.

The non-enzymatic, acid-catalyzed cyclization is evident from control incubations containing no versicolorin B synthase. The second-order rate constant for this bimolecular process (k_{cyc}), governed by substrate and proton concentration, was estimated to be 16 M^{-1} min^{-1} from these control experiments by fitting the data to eq 1

$$v = k_{cyc}[S][H^+] \quad (1)$$

where [S] and [H^+] represent the substrate and proton concentrations, respectively. The data indicate that cyclization increasingly occurs at low pH, underscoring the importance of distinguishing between chemical and enzymatic conversion under these conditions. The acid-catalysis of the

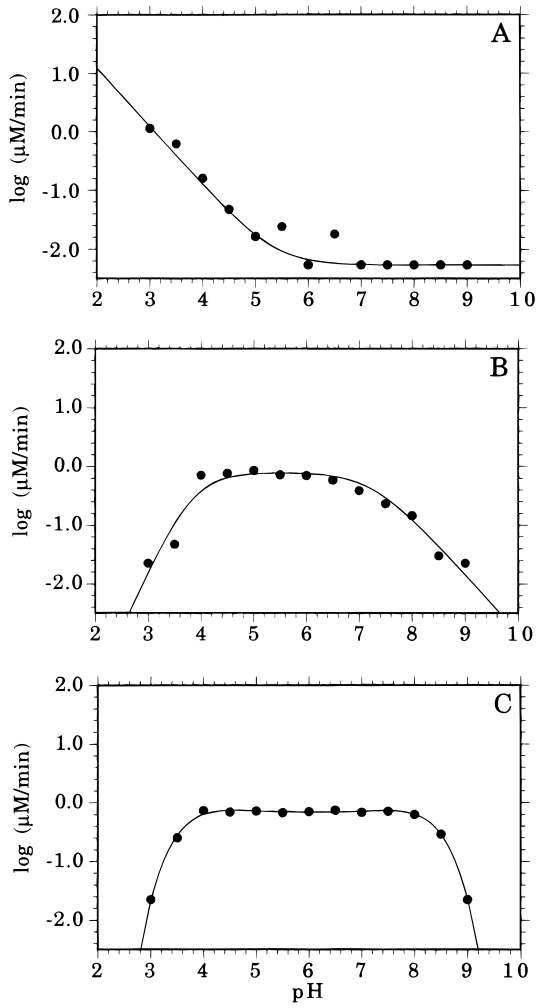


FIGURE 3: Effect of pH on rate of versicolorin B formation. A, non-enzymatic cyclization; B, VBS-catalyzed cyclization; C, VBS stability.

reaction may also suggest an important mechanistic feature of the enzyme-catalyzed reaction.

The data for the enzymatic reaction represented in Figure 3 have been corrected for the background acid-catalyzed cyclization for each pH studied. Interestingly, the ability of versicolorin B synthase to stably catalyze the reaction over a broad range of pH values, as low as pH 4.0, is observed. This profile is consistent with two well-separated ionizations occurring within the enzyme−substrate complex that effect activity (Tipton & Dixon, 1979). It is possible to estimate the acid dissociation constants associated with these ionizations through analysis of the changing velocities. For an ideal case, in which protons act as non-competitive effectors of the enzyme, eq 2 expresses the dependence of the maximal rate of reaction (V) on proton concentration ([H^+]) given that the two acid-dissociation constants are sufficiently different ($K_a \gg K_b$) (Alberty & Massey, 1955; Waley, 1953). The term k[E] represents the proton-independent maximal rate, which is dependent on the quantity of enzyme.

$$V = k[E]/(1 + [H^+]/K_a + K_b/[H^+]) \quad (2)$$

The drastic decline in activity observed between pH 4.0 and 3.0 is inconsistent, however, with only one ionization occurring in this range. The slope of this decrease is greater than +1 as is predicted from eq 2, although this phenomenon may be due to a lack of accuracy in measuring the enzymatic

rate over a high competing chemical cyclization or even some structural instability of the protein under these extreme conditions. Two ionizations are assumed to be the cause of the sharp decrease in rate since triplicate assays and the protein pH−stability served as controls for the former two possibilities. If the enzyme−substrate complex is experiencing two protonations at this pH range, and their pK_a values are closely related, then the proton concentration dependence of the initial, maximal rate can be expressed by eq 3

$$V = k[E]/(1 + [H^+]/K_a + [H^+]^2/K_a^2 + K_b/[H^+]) \quad (3)$$

where K_a now represents the average molecular dissociation constant of the two ionizations which occur in the acidic range (Cleland, 1979). Using a nonlinear regression analysis, the data for the enzyme-catalyzed reaction were fitted to the logarithmic form of eq 3 to obtain k[E], K_a, and K_b values. The curve is displayed in Figure 3. The average pK_a value responsible for the sharp decline in activity of VBS was found to be 3.7 ± 0.1. This value is consistent with carboxylate residues such as glutamate or aspartate. The slope of this curve may indicate the presence of up to two acidic amino acid residues necessary for catalysis. This observation would explain the high degree of reactivity of the enzyme at low pH values. It is of interest, in light of the potential mechanisms involved in the cyclization (McGuire & Townsend, 1993), to note that many glycosidases use such residues in the hydrolysis of acetal linkages, either through direct displacement or as a means of ionizing water within their active sites (Cabezas et al., 1983; Sinnott, 1990). Similar speculation about the other acid dissociation constant obtained from this analysis, 7.3 ± 0.1, would indicate the participation of an amino acid with a perturbed ionization, possibly histidine (pK_a = 5.5−7.0) or cysteine (pK_a = 8.0−8.5). The slope of the pH−stability profile indicates the presence of only one ionizable amino acid residue in this pH range. Since, however, as the maximal velocity estimates can only provide information concerning the enzyme−substrate complex, the substrate must also be considered. Versiconal hemiacetal (**8**) undergoes a dramatic color change from orange to purple at alkaline pH which allows determination of the pK_a for this process spectrophotometrically using the method of Albert and Serjeant (1984). Analysis of versiconal hemiacetal in a constant ionic strength buffer by UV spectroscopy at 319 nm at six different pH values (pH 5.9−7.5) revealed the acid dissociation constant (pK_a) to be 6.8 ± 0.1, quite a low value for a phenol. These data could be interpreted to suggest that the C-6 phenol **12** loses its proton to give an extended chromophore as shown in Scheme 2. Deprotonation *para* to the C-9 anthraquinone carbonyl is likely favored by hydrogen bonding from the adjacent *peri*-hydroxyl groups at C-1 and C-8. This interaction can be visualized to give the uniquely stabilized tautomer, enolate **13**. The observed ionization of the enzyme−substrate complex could, therefore, be ascribed to deprotonation of the substrate as well as to a cysteine or histidine residue of VBS. If the mode of binding of versiconal hemiacetal with VBS involves an association between a hydrophobic region of the protein and the anthraquinone, the development of a negative charge would decrease this affinity. This, in turn, would affect the overall rate of catalysis in a manner consistent with that observed.

Kinetic Analysis. Analyses of the effect of changing substrate concentrations on initial velocities was used to determine the kinetic parameters of V_{max} and K_m for versicolorin B synthase. The selection of concentrations was made after preliminary experiments gave a rough estimate of K_m. These were assayed in triplicate with a dilute active solution of purified VBS to obtain initial rate values expressed as μM/min. The results were fit directly to the Michaelis−Menten equation using nonlinear regression. The results, including the calculated curve, are graphically represented in Figure 4. The V_{max} obtained under these conditions was 0.14 ± 0.01 μM/min, and the K_m, indicated by the racemic substrate concentration at half-maximal velocity, was found to be 2.4 ± 0.3 μM. The value of V_{max} provides a means to estimate k_{cat}, the enzymatic second-order rate constant, based on the quantity of purified protein used, providing a turnover number of 2.5 s^{-1} for each subunit of VBS.

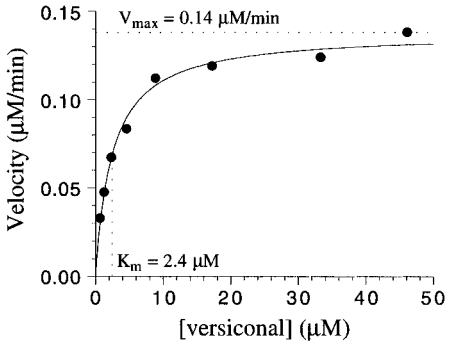
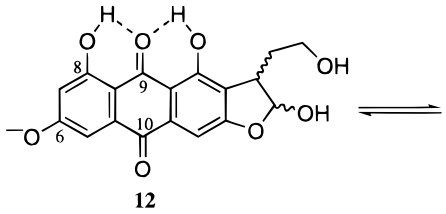


FIGURE 4: Nonlinear determination of K_m and V_{max} of VBS.

Scheme 2



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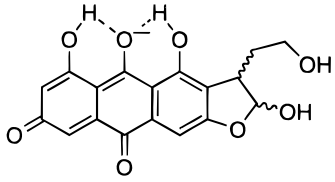

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The value of the Michaelis constant is quite low for a secondary metabolic enzyme and indicates a high affinity between the enzyme and substrate. This fact, in addition, serves to validate the previous assays that were run at concentrations approximating 100 μM, which thereby can be considered as operating under saturating conditions.

One unaddressed feature of the reaction system concerned the stereochemistry of the product. In the above kinetic treatment, all of the substrate was assumed to be potentially reactive within the context of enzyme catalyzed cyclization. This is not necessarily true. Versiconal hemiacetal (**8**) and metabolites **6** and **7**, which possess the cyclic hemiacetal, are isolated as racemates (Cox et al., 1977; Steyn et al., 1979; Townsend et al., 1988); however, all of the aflatoxin biosynthetic intermediates subsequently formed are optically active. Since hydroxyversicolorone (**6**, HVN) is in turn formed from averufin (**5**, AVR), which exists as one enantiomer (Koreeda et al., 1985), it is unclear whether the

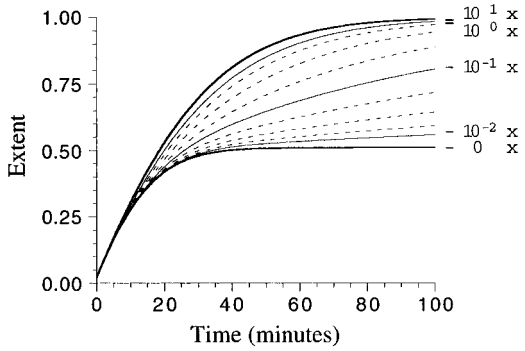


FIGURE 5: Theoretical time course curves for VBS as a function of racemization rate constant (k_{rac}). The conditions illustrated represent a racemic substrate concentration of 2.14 μM, an initial product concentration of 0.044 μM, and a V_{max}/K_m value of 0.13 min^{-1}. The various values of k_{rac} are provided as a function of V_{max}/K_m. The solid lines represent single order of magnitude variation, while the dashed lines represent one-quarter of that magnitude (*i.e.*, $10^{-0.25}$, $10^{-0.50}$, $10^{-0.75}$).

rapidity of racemization plays a significant role physiologically. Initial studies of the *A. parasiticus* cell-free extract system capable of the transformation of racemic versiconal hemiacetal acetate into versicolorin B indicated that, after 4 h of incubation at a concentration greater than 100 μM, pH 7.5, complete conversion to product had taken place (Brobst, 1989). This phenomenon suggests that racemization occurs relatively rapidly in buffered aqueous solutions and that versicolorin B synthase may play a role in selectively processing one enantiomer to (−)-versicolorin B (**9**). Figure 5 illustrates the effects that various theoretical rates of interconversion between the two enantiomers (racemization) of the substrate would have on the overall enzyme-catalyzed cyclization reaction. Within the context of the enzymatic transformation, racemization is seen to dictate the rate of conversion of *total* concentrations of versiconal hemiacetal (**8**) to versicolorin B (**9**).

In order to determine the precise role of racemization in this biosynthetic system, an analysis of VBS was initiated to ascertain the relative rate of racemization, as compared to the enzyme-catalyzed cyclization to tetrahydrobisfuran. The system is unusual in that a dynamically racemic substrate is transformed into an optically active product. It was hoped that polarimetry would yield a means of measuring the reaction in terms of the appearance of an optical rotation arising from product formation. The uniqueness of the VBS system could not be exploited in this way owing to absorbance by, and the relative insolubility of, both the substrate and product in the visible light range. Low solubility was a further hindrance to possible exchange experiments using ^{1}H NMR to monitor the uptake of deuterium isotope by versiconal hemiacetal from D_2O.

Therefore, measurement of the racemization rate was addressed through the analysis of conversion kinetics using the sensitive radiochemical enzymic assay. Simple analysis of the time-dependent production of versicolorin B should reveal whether the entire quantity of substrate is consumed, or just half of the total as would be consistent with no interconversion between enantiomers. Intermediate rates of racemization may also be calculated from such a progress curve in a manner similar to that used to analyze the anomeric specificity of carbohydrate-utilizing enzymes (Benkovic, 1979). In order to circumvent the potential problem of instability of versicolorin B synthase through time, it is possible to incubate small quantities of substrate. Although the rate of conversion is slowed by concentrations of versiconal hemiacetal near its K_m value, small concentrations of enzyme are still capable of completely converting this amount of substrate within short time periods (<100 min). Since the percentage of substrate remaining is the important parameter, dilute concentrations can be utilized, so long as changes in velocity due to diminishing quantities of versiconal hemiacetal are taken into account.

Furthermore, the kinetic parameters which have been determined above have assumed that both antipodes of the racemate act as substrate. In fact, only 50% of the total initial concentration possesses the same absolute configuration as (−)-versicolorin B (**9**). It is assumed, therefore, that one enantiomer of versiconal hemiacetal acts as substrate while the other is potentially free to act as a competitive inhibitor of the enzyme. Accordingly, the Michaelis constant was re−evaluated through analysis of the experimental data described above, using the expanded initial rate equation (eq 4; see Appendix)

$$v = V_{max}[S]/(K_m + K_m[S']/K_S' + [S]) \quad (4)$$

where [S] is the reactive isomer, [S′] is the non-reactive antipode, and $K_{S'}$ is its inhibition constant. K_m was found to be 1.04 μM; the inhibition constant was estimated to be 15 μM, which is more than an order of magnitude greater than K_m. Thus competitive inhibition by S' is minimal but can still be taken into account in further studies.

Another aspect of this system to be considered was the effect of the product on the enzymatic cyclization rate. Within the scope of the time course study proposed the amount of product will far exceed that of remaining substrate after extended time periods. If the affinity of VBS for versicolorin B is comparable to that for versiconal hemiacetal, the enzyme will become severely inhibited, preventing complete turnover of substrate. This possible competitive inhibition by product can be easily studied through assays of the enzyme in the presence of varying quantities of versicolorin B. The low solubility of the product limited the study to concentrations of 50 μM or less. To estimate the inhibition constant of versicolorin B, the rate expression in eq 5 was used

$$v = V_{max}[S]/(K_m + K_m[S']/K_{S'} + K_m[P]/K_P + [S]) \quad (5)$$

where K_P represents the inhibition constant for the product, P. This constant was estimated through analysis of the fractional inhibition (i) (Segel, 1975) as defined in eq 6

$$i = 1 - v_p/v_0 = [P]/([P] + K_p(1 + [S']/K_{S'} + [S]/K_m)) \quad (6)$$

where v_P and v_0 represent the rates of conversion in the presence and absence of versicolorin B, respectively. The values of the $1 - (v_P/v_0)$ term were calculated from duplicate assays of VBS in 2 μM (±)-versiconal hemiacetal with six different versicolorin B concentrations (0.55−55 μM) as compared to the identical assays with no product added (Figure 6). Nonlinear regression of the data to eq 6 provided an estimate of K_p of 11 μM, again a full order of magnitude above K_m. Therefore, product inhibition will play only a minimal, but calculable, role in the progress studies of versicolorin B synthase.

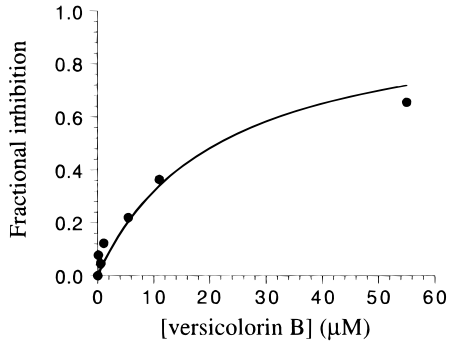


FIGURE 6: Estimation of the competitive inhibition constant of versicolorin B for VBS. Analysis of the fractional inhibition (eq 6) by the product translates into a K_P of 11 μM for the assay conditions employed.

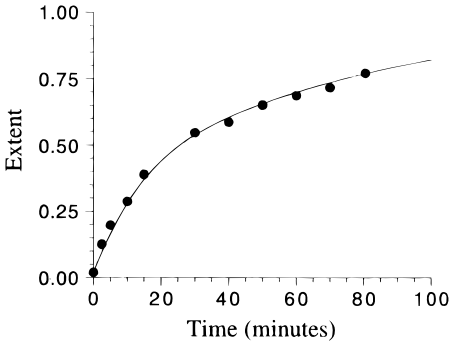


FIGURE 7: Time course study of VBS. The points represent the average of triplicate assays of VBS with 2.14 μM versiconal hemiacetal. The amount of versicolorin B is expressed as a fraction of the initial quantity of substrate and product or as the extent of the reaction. The curve was generated as described in the Appendix using a racemization rate constant (k_{rac}) of 0.015 min^{-1}.

With each of the parameters of the system addressed quantitatively, the time-dependent, enzymatic production of versicolorin B was monitored to determine the relative rate of racemization. Identical, triplicate assays were performed in 10 mM potassium phosphate (pH 6.5) buffer with 20% glycerol containing purified versicolorin B synthase and 2.1 μM (±)-versiconal hemiacetal (**8**) to obtain eleven time points. Control assays showed that virtually no background cyclization occurred in the 80 min of incubation. The amount of versicolorin B produced in each assay is expressed as a fraction of initial total substrate ("Extent") in Figure 7. The progress of versicolorin B synthase is biphasic under these conditions, indicating that racemization occurs in aqueous systems and can limit net flux to product. Thus, racemization is rapid enough to allow complete conversion of versiconal hemiacetal to versicolorin B as was initially experienced (McGuire et al., 1989) but remains slow enough to allow kinetic differentiation between processing of the two substrate sources, S and S′, when the ratio of enzyme/substrate(s) is high enough. That is, the rate of overall conversion becomes limited by the racemization rate and can be evaluated as described in the Appendix. Once the majority of the reactive isomer, S, has been consumed, its concentration is maintained at steady-state levels. From this point, the extent of reaction through time will display first-order kinetics dependent on the rate of racemization. The overall rate constant (k_{obs}) governing the consumption of S′ to form P was calculated from the extent of reaction within the time frame of 40−80 min (Figure 8) and found to be 0.014 min^{-1}. Through the relation outlined in eq 16 and using the value of V_{max}/K_m (0.13 min^{-1}) to denote the

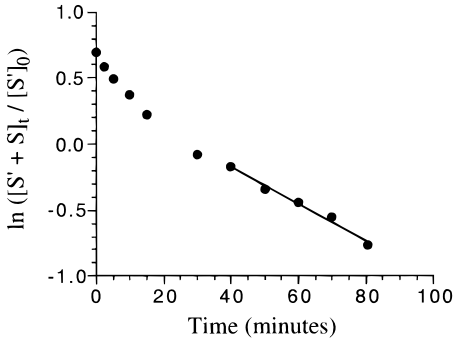


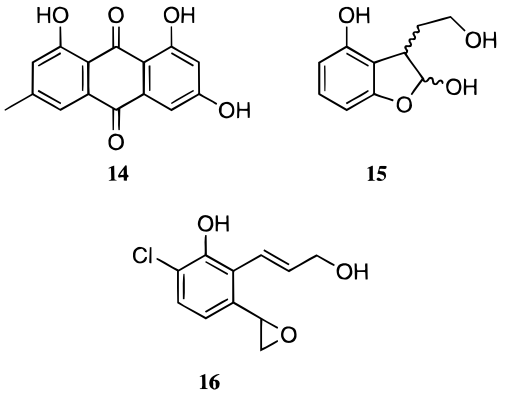
FIGURE 8: Calculation of the first-order rate constant, k_{obs}, for the formation of versicolorin B from the uncatalyzed enantiomer of versiconal hemiacetal. The data were obtained from the experiment shown in Figure 7. The value of $[S']_0$ was taken as 1.07 μM. The rate constant was taken from the slope of the line formed from the time points within the 40−80 min range as shown.

apparent first-order rate constant (k_{enz}) of the enzymatic conversion of S to P under low concentrations of S, the value of the racemization rate constant (k_{rac}) was found to be 0.015 min^{-1}. Figure 7 demonstrates the theoretical progress curve utilizing the calculated racemization parameter, in conjunction with the experimental data. The fit is quite good considering the quality of the required steady-state assumption. Subsequent experiments performed under similar conditions corroborate this value, including an experiment in which the active enzyme concentration was almost half that used previously (k_{enz} = 0.076 μM/min), and yet the racemization remained similar (k_{rac} = 0.014 min^{-1}); thus, the inversion of the stereochemistry of the substrate is not an enzyme-mediated event.

The rate of racemization, despite being an order of magnitude less than the enzyme-catalyzed cyclization of versiconal hemiacetal under the conditions of the assay shown, is ample enough to provide total conversion of both enantiomers into product. Interpretation of the role of this process *in vivo* is difficult without knowing the concentration levels of enzyme and substrate within the fungus. Since racemization is an apparent unimolecular process, the rate of stereochemical interconversion can continue to increase even after the enzymatic reaction is experiencing saturation kinetic conditions under high substrate concentrations. However, it is unlikely that the quantity of versiconal hemiacetal ever exceeds low steady-state levels given the relatively low K_m of VBS. At these low concentrations of substrate the quantity of enzyme would dictate the extent to which versiconal hemiacetal racemizes. Regardless of the stereospecificity of the enzymes that catalyze the reactions involving the precursor metabolites hydroxyversicolorone (**6**) and versiconal acetate (**7**), it should be recognized that versicolorin B synthase plays a crucial role by reintroducing stereochemical control in the biosynthesis to set the absolute configuration of the bisfuran ring system.

Inhibition and Attempted Inactivation of VBS. Knowing now that versicolorin B synthase selects the correct stereoisomer between the (+) and (−)-antipodes of versiconal hemiacetal (**8**) to cyclize to (−)-versicolorin B (**9**), and that the ^{18}O-label from the primary alcohol of the former appears without loss in the tetrahydrobisfuran (McGuire & Townsend, 1993) of the latter (Scheme 1), suggested the presence of an enzyme active site acid to catalyze this cyclization. The pH−rate profile of VBS supported this mechanistic inference and the involvement of a group to assist dehydration of the

Chart 2



Scheme 3

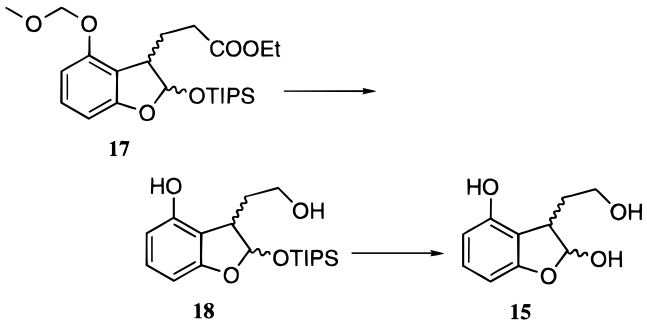


Table 3: VBS Inhibitor Screening

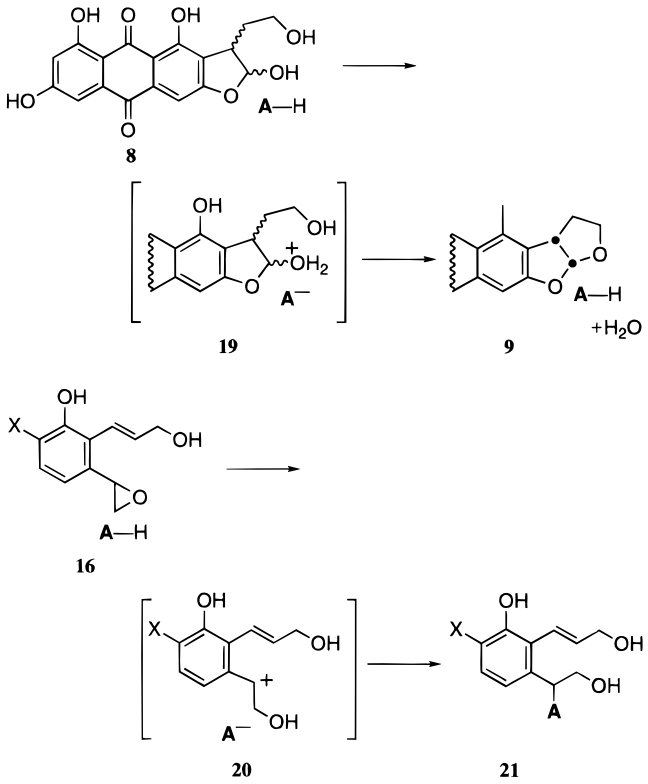
compound	concentration (μM)	relative activity
2-hydroxyphenylacetic acid lactone	2000	1.03
2,5-dihydroxyphenylacetic acid lactone	2000	0.97
emodin (**14**)	750	1.01
versicolorin B (**9**)	15	0.50
diol (**15**)	50	0.71

hemiacetal. With the aim to prepare a covalent inactivator of this enzyme, ideally to react specifically at this acidic residue, several simple structures were tested as potential inhibitors of the VBS-catalyzed cyclization. Among those examined were 2-hydroxyphenylacetic acid lactone, 2,5-dihydroxyphenylacetic acid lactone, emodin (**14**), versicolorin C (**9**), and hemiacetal diol **15**, a close structural analogue of the right-hand half of the normal substrate (Chart 2).

While most of these compounds were commercially available, versicolorin C [the racemic form of versicolorin B (**9**)] had been prepared earlier (Graybill et al., 1989), and the hemiacetal diol **15** was readily accessible in three steps from an intermediate in that synthesis as illustrated in Scheme 3. The differentially protected ester **17** (Graybill et al., 1989) was treated with $AlCl_3$ to remove the methoxymethyl protecting group and reduced to give diol **18**. Desilylation readily took place in the presence of *n*-Bu_4NF to give **15**.

The compounds were rapidly surveyed for their relative extents of inhibition as a function of concentration. Like the hydroxyphenylacetic acid lactones, most of these had no effect on the cyclization reaction. Notably, emodin (**14**) containing the trihydroxylated anthraquinone analogous to the substrate showed no inhibition (Table 3) while the product of the reaction, versicolorin B (**9**), did. Most important among these experiments, **15**, which mimics the C-ring and side chain of the substrate **8**, was found to

Scheme 4

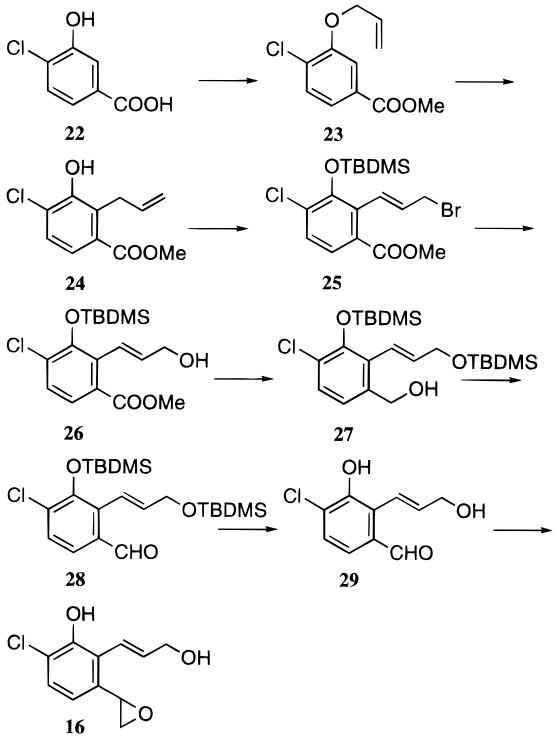


decrease the efficiency of the normal reaction. More detailed investigation of the inhibitory behavior of **15** indicated a cleanly competitive pattern with a K_i of 76 ± 19 μM. These simple experiments suggested that substrate recognition by VBS was less dependent on the anthraquinone nucleus approximated by emodin (**14**) but driven more by the side chain and first aromatic ring. Heartened by the possibility of using a simple benzenoid framework for the design of a potential irreversible inactivator, the benzylic epoxide **16** was selected as a target for synthesis.

The choice of **16** stems from the successful use of distantly related epoxides by Ganem (Tong & Ganem, 1988) and Legler (Legler & Herrchen, 1981) to inhibit glycosidases. The rationale for the specific case of VBS is illustrated in Scheme 3. The normal reaction is shown at the top where a hypothetical active site is proposed to protonate the hemiacetal to give transient intermediate **19**. This unstable species may be visualized to dehydrate to an oxonium ion or suffer displacement by the primary alcohol (*) to give versicolorin B (**9**). Analogously, potential inactivator **16** could be envisioned to undergo similar protonation to give benzylic cation **20**, whose stability could be modulated by the nature of X (in this case X = Cl) and which could trap the conjugate base, A−, of the enzyme to give covalent adduct **21**. The *trans*-double bond of the propenol side chain should be geometrically forbidden from trivial closure of the reactive intermediate (Scheme 4).

Fischer esterification of 3-hydroxyl-4-chlorobenzoic acid (**22**) and Claisen rearrangement of the allyl ether **23** gave the terminal olefin **24** in 75% yield for these steps (Scheme 4). Oxidation of the three-carbon side chain to the allylic alcohol present in **16** was plagued by ready lactonization to the adjacent ester. The difficulties encountered in several ionic pathways to this transformation were overcome by free radical bromination of the silyl-protected phenol to give the cinnamyl bromide **25** (71%) (Casillas, 1996). Further radical

Scheme 5



reaction of this bromide by the method of Nakamura et al. (1991) with molecular oxygen and 2 equiv of tributyltin hydride (*n*-Bu_3SnH) gave the allylic alcohol **26** (67%) largely by way of the intermediate hydroperoxide. Protection of the allylic alcohol and reduction of the ester with Dibal-H proceeded smoothly to afford **27**. Mild oxidation to the aldehyde **28** was achieved using the method of Martin (Martin & Smith, 1964). While it was intended to prepare the key epoxide by addition of trimethylsulfonium ylide, followed by removal of the silyl protecting groups, the allylic TBDMS was unexpectedly found to be extremely resistant to conventional deprotection protocols. Finally, the procedure of Batten et al. (1980) succeeded in removing the TBDMS groups from **28** to yield **29**, which was directly reacted to the desired **16** in the presence of excess trimethylsulfonium ylide (Scheme 5).

The epoxide **16** was initially assayed for its behavior as an inhibitor/activator at 25, 250, and 2500 μM in the same fashion as for the compounds shown in Table 2. No effect on the conversion of versiconal hemiacetal to versicolorin B was observed. To test for slow binding and inactivation, **16** was similarly incubated with VBS at 2.50 mM for 1 h for 10 min intervals prior to activity assay. Unfortunately, no inactivation of VBS was observed.

CONCLUSIONS

The failure of styrene oxide **16** to covalently modify versicolorin B synthase was disappointing, but perhaps notunexpected given the geometric constraints of this potential inactivator to reaction at the active site. Nonetheless, the unusually broad pH−rate profile and the involvement of a group ionizing at pH 3.7 ± 0.1 evidenced in these studies are in keeping with the course of isotope label (*) in a dehydrative cyclization path catalyzed by an acidic group of the enzyme (Scheme 1). Despite the marginal efficiency of earlier efforts to purify VBS (Anderson & Green, 1994; Lin & Anderson, 1992; Yabe & Hamasaki, 1993), its stability at low pH was key to the successful method described here. Preparative isoelectric focusing played an essential role to obtain homogeneous protein in good yield, which appeared to be a dimer of identical subunits of 78 000 Da. No cofactors are required for catalysis, and no bound cofactors were evident from UV spectroscopy. Kinetic characterization of this enzyme revealed an unusually low K_m, *ca.* 1 μM, for a secondary metabolic enzyme. VBS has been demonstrated to selectively turn over one of two configurationally dynamic antipodes of a racemic substrate **8** for cyclization to the optically active product (−)-**9**. By varying the relative amount of protein to substrate, it was possible to make chemical racemization of this stereochemically labile hemiacetal rate-limiting and, hence, measurable—a process not readily quantified by other means and not apparently catalyzed by the enzyme itself.

Intermediates **6**−**8** (Scheme 1) are all isolated as racemates. VBS plays the important role of re-establishing asymmetry in the biosynthesis to set the absolute configuration of the critical bisfuran in **9** that ultimately appears in aflatoxin B_1 (**1**). It is for this reason that VBS was chosen for mechanistic analysis and as a target enzyme for inhibitor design. Metabolic activation of the electron-rich furano vinyl ether of **1** generates the *exo*-epoxide **2** then giving a highly reactive entity correctly matched in its absolute stereochemistry to lead, after intercalation, to covalent reaction at the N-7 of guanine residues. The carcinogenic properties of aflatoxin B_1, therefore, depend on the stereochemical choice made by VBS between the enantiomers of versiconal hemiacetal **8**.

Complementation of two aflatoxin pathway mutants (Skory et al., 1993; Skory et al., 1992) and purification of a transmethylase (Bhatnagar et al., 1988) have led to cloning of three genes involved in the biosynthesis of the mycotoxin (Yu et al., 1995). Mapping experiments have suggested strongly that these genes are linked and in turn have led to extensive sequencing of the flanking DNA to identify the polyketide synthase and two fatty acid synthases believed responsible for initiation of this biosynthetic pathway (Mahanti et al., 1996). Specific gene disruption (Mahanti et al., 1996) and biochemical experiments (Watanabe et al., 1996) support these roles. The gene encoding VBS has been cloned, mapped and sequenced and found to lie 3.3 kbp from the methyltransferase identified above (Silva et al., 1996). This observation expands the dimensions of the emerging aflatoxin biosynthetic gene cluster and supports the view that the entire pathway will be encoded in a large, contiguous stretch of the genome encompassing *ca.* 60 kbp.

APPENDIX

To mathematically express the possible competitive inhibition by an unreactive enantiomer (S′) of the substrate (S), the Michaelis−Menten equation can be expanded as in eq 4 where $K_{S'}$ represents the inhibition constant for the anomer with the incorrect stereochemistry. Taking the reciprocal of eq 4 and multiplying by V_{max}

$$V_{max}/v = K_m/[S] + K_m[S']/K_{S'}[S] + [S]/[S] \qquad (7)$$

yields a relationship analogous to the Lineweaver−Burk double-reciprocal plot between inverse velocity and inverse substrate concentration. This relationship can be further simplified through the realization that, at initial rates of

reaction, the two concentrations of the racemate ($[S]_0$ and $[S']_0$) are equal, so that the equation

$$V_{max}/v = K_m/[S] + K_m/K_{S'} + 1 \quad (8)$$

reveals a linear relationship between the term on the left, and the inverse of the true substrate concentration. The slope of this line reveals K_m and the intercept can be expressed in terms of $K_{S'}$.

Given the system described in eq 9, wherein a substrate (S) is in equilibrium with its enantiomer (S′),

$$S \underset{k_{rac}}{\overset{k_{rac}}{\rightleftharpoons}} S' \xrightarrow{k_{enz}} P \quad (9)$$

the rate of interconversion of the two species is governed by the first-order rate constant k_{rac}. Introduction of an enzyme capable of selectively processing S to form product (P) renders the rate expression for the time-dependent production of P and consumption of S as described in eqs 10 and 11, respectively

$$d[P]/dt = k_{enz}[S] \quad (10)$$

$$-d[S]/dt = k_{enz}[S] + k_{rac}[S] - k_{rac}[S'] \quad (11)$$

where k_{enz} represents the apparent first-order rate constant of the enzyme.

If the racemization rate is less than that of the enzymatic reaction ($k_{rac} < k_{enz}$), then the flux of material will proceed directly from S′ to produce P once S has attained steady-state concentrations. Under these conditions the net rate of change in S concentration will be zero. Thus, substituting zero for the term $-d[S]/dt$ in eq 11 yields a time-dependent expression for [S] in eq 12

$$[S] = (k_{rac}/(k_{rac} + k_{enz}))[S'] \quad (12)$$

in terms of [S′]. Substitution of this term for [S] in eq 10 provides eq 13

$$d[P]/dt = (k_{rac}k_{enz}/(k_{rac} + k_{enz}))[S'] \quad (13)$$

which shows that the formation of product, once steady-state conditions have been achieved, will exhibit first-order kinetics in terms of S′, with the observed rate constant (k_{obs}) being composed of the terms k_{enz} and k_{rac} as in eq 14.

$$k_{obs} = k_{rac}k_{enz}/(k_{rac} + k_{enz}) \quad (14)$$

Unfortunately, only product formation, and thereby total substrate ([S] + [S′]) consumption, is detectable through time. If the concentration of S has achieved steady-state concentrations so that $[S] \ll [S']$, the total substrate concentration will approach that of S′ alone as in eq 15

$$[S'] \approx [S'] + [S] = [S']_0 + [S]_0 - [P] \quad (15)$$

where $[S]_0 + [S']_0$ represents the initial concentration of racemic substrate. An estimate for k_{enz} is obtained through the term V_{max}/K_m for enzymatic reactions run under low substrate concentrations, as is the case when [S] is under steady-state control. Therefore a value for k_{rac} is found from rearrangement of eq 14 to obtain eq 16

$$k_{rac} = k_{obs}k_{enz}/(k_{obs} - k_{enz}) \quad (16)$$

once k_{obs} is determined.

In order to calculate the theoretical time-dependent production of product, the enzymatic rate expression is defined as in eq 17

$$d[P]/dt = V_{max}[S]/(K_m + K_m[S']/K_{S'} + K_m[P]/K_P + [S]) \quad (17)$$

which is similar to eq 4 but has an additional term in the denominator to account for product inhibition (K_P = product inhibition constant). This expression is composed of three terms which vary with time, [S], [S′], and [P]. The rate equations governing the concentrations of S and S′, as defined by eq 9, are shown in eqs 18 and 19, respectively.

$$d[S]/dt = -d[P]/dt + k_{rac}[S'] - k_{rac}[S] \quad (18)$$

$$d[S']/dt = k_{rac}[S] - k_{rac}[S'] \quad (19)$$

An iterative computer algorithm was developed to monitor these changes in [S], [S′], and [P] with incremental changes in time within the constraints of eqs 17−19.

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