Trichodiene Synthase. Probing the Role of the Highly Conserved Aspartate-Rich Region by Site-Directed Mutagenesis†

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ABSTRACT: Trichodiene synthase catalyzes the cyclization of farnesyl diphosphate to the sesquiterpene hydrocarbon trichodiene. The enzyme normally requires a divalent cation, Mg^{2+}, which can be substituted by Mn^{2+}. Trichodiene synthase from *Fusarium sporotrichioides* has a highly conserved aspartate rich region, aa 100−104 (DDSKD). Three mutants were constructed by site-directed mutagenesis in which each aspartate residue was individually replaced by glutamate. The mutants were each overexpressed and purified to homogeneity. The importance of Asp100 and Asp101 for catalysis was established by the observation of an increase in K_m as well as a reduction in k_{cat} in the corresponding Glu mutants. Replacement of the Asp104 residue with Glu had little effect on either K_m or k_{cat}. All three mutants produced anomalous sesquiterpene products in addition to trichodiene when incubated with farnesyl diphosphate. Interestingly, when Mg^{2+} was replaced by Mn^{2+} in the incubation buffer, the k_{cat}/K_m of both wild type trichodiene synthase and the D104E dropped significantly, while those of the other two mutants were not much affected. The proportion of anomalous products increased significantly when the D100E and D101E mutants were incubated in the presence of Mn^{2+}. These observations all lend weight to the proposal that the aspartate residues mediate substrate binding by chelation of the divalent metal ion. Asp100 and Asp101 appear to play a relatively more important role than Asp104.

Trichodiene synthase (TS)[1] catalyzes the cyclization of farnesyl diphosphate (FPP, **1**) to trichodiene (**2**), the parent sesquiterpene hydrocarbon of the trichothecane family of antibiotics and mycotoxins (Cane et al., 1981). Trichodiene synthase has been extensively studied with respect to mechanism, enzymology, and molecular genetics (Cane, 1990; Cane & Yang, 1994; Cane et al. 1995a,b). Extensive mechanistic studies have lent strong support for the cyclization mechanism illustrated in Scheme 1 according to which FPP undergoes an initial ionization and rearrangement to the tertiary allylic isomer, (3*R*)-nerolidyl diphosphate [(3*R*)-NPP, **3**], followed by a second ionization and electrophilic cyclization involving the central double bond (Cane et al., 1985, 1990, 1992; Cane & Ha, 1988). Further cyclization of the bisabolyl cation (**4**) coupled with a series of hydride shifts and methyl migrations leads ultimately to formation of trichodiene. Typical of all terpenoid synthases, a major determinant of the specificity of trichodiene synthase is believed to be the precise folding of the substrate, FPP, at the cyclase active site. Under normal circumstances, the numerous cationic intermediates, which are never released from the active site, much be protected from premature quenching, either by reaction with external nucleophiles such as water, or by interaction with the protein itself. We recently described studies in which alteration by site-directed mutagenesis of an arginine-rich region of the presumptive active site led to the formation of multiple sesquiterpenes, evidently resulting from premature deprotonation of the normally cryptic cationic cyclization intermediates (Cane & Xue, 1996). We now report investigations in which site-directed mutagenesis has been used to clarify the role of the conserved aspartate residues in binding and catalysis by trichodiene synthase.

Trichodiene synthase has been isolated from a variety of fungal sources, including *Trichothecium roseum* (Evans & Hanson, 1976; Cane et al., 1981). *Fusarium sambucinum* (*Gibberella pulicaris*) (Hohn & Beremand, 1989a), and *Fusarium sporotrichioides* (Hohn & VanMiddlesworth, 1986). The *F. sporotrichioides* cyclase has been purified to homogeneity and found to be a homodimer of M_r 45 000 subunits, as determined by SDS−PAGE. The corresponding structural gene has been cloned (Hohn & Beremand, 1989b; Hohn & Plattner, 1989) and overexpressed (Cane et al., 1993) in *Escherichia coli*. Comparison of the sequences of the *F. sporotrichioides* and *G. pulicaris* enzymes showed that they have an 89% identity at the nucleotide level and a 96% identity at the amino acid level (Hohn & Desjardins, 1991). Additional TS genes have been sequenced from *Gibberella zeae* (Proctor et al., 1995). *Fusarium poae* (Genbank U15658; Fekete, C., Giczey, G., Papp, I., Taborhegyi, E., Szabo, L., and Hornok, L., unpublished), and *Myrrothecium roridum* (S. Trapp, T. Hohn, and B. Jarvis, personal communication). Although all these genes have high degrees of mutual sequence similarity, none of the TS genes shows any significant sequence similarity to any other known genes or proteins. The TS enzymes do contain two short, highly conserved sequence motifs of potential functional significance. The basic amino acid-rich sequence, DRRYR, is found in the *F. sporotrichioides* TS, while the closely related

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[1] Abbreviations: FPP, farnesyl diphosphate; IPTG, isopropyl β-thiogalactopyranoside, GC−MS, capillary gas chromatography−mass spectrometry; LBA, Luria−Bertani ampicillin; PCR, polymerase chain reaction; SDS−PAGE, sodium sulfate−polyacrylamide gel electrophoresis; TS, trichodiene synthase.

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Scheme 1: Cyclization of FPP to Trichodiene

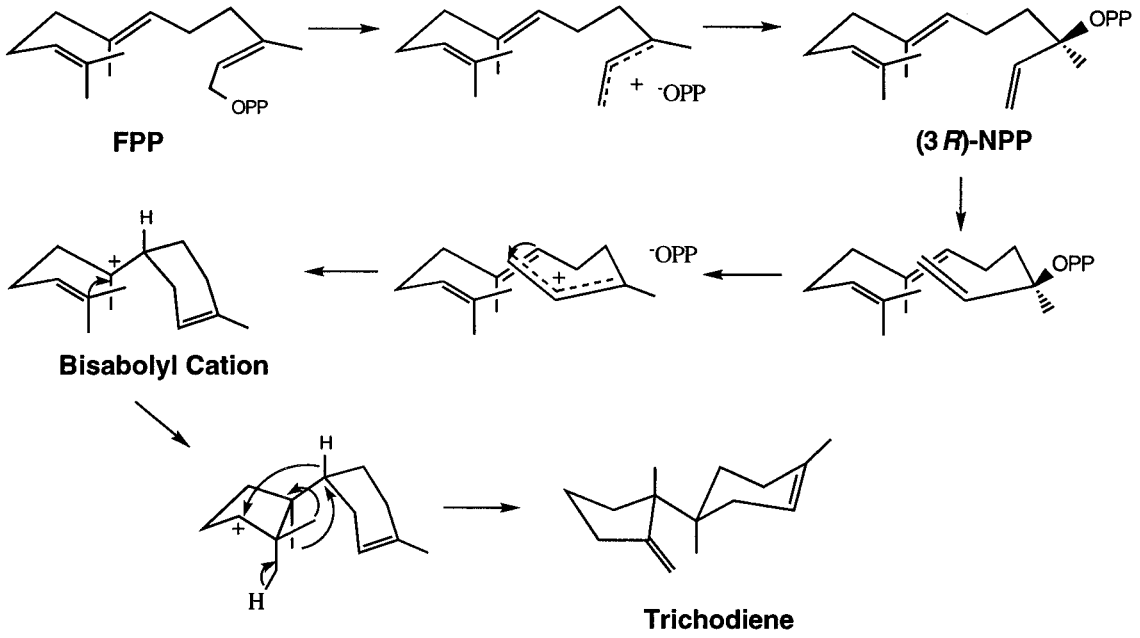


Table 1: Aspartate-Rich Domains in Terpenoid Synthases

synthase	sequence	reference
trichodiene synthase		
F. sporotrichioides	VLDDSKD	Hohn & Beremand, 1989
G. pulicaris	VLDDSSD	Hohn & Desjardins, 1991
G. zeae	VLDDSSD	Proctor et al., 1995
F. poae	VLDDSSD	Genbank Accession No. U15658
M. roridum	ILDDSED	S. Trapp, T. Hohn, and B. Jarvis, personal communication
pentalenene synthase	FLDDFLD	Cane et al., 1994
aristolochene synthase	LIDDVLE	Proctor & Hohn, 1993
epi-aristolochene synthase	IVDDTFD	Facchini & Chappell, 1992
cadinene synthase	IVDDTYD	Chen et al., 1995
casbene synthase	LIDDTID	Mau & West, 1994
limonene synthase	VIDDIYD	Colby et al., 1993

sequence, DHRYR, is found in both the *F. poae* and *G. pulicaris* enzymes, respectively. The important roles of Arg304, Tyr305, and Arg 306 in TS from *F. sporotrichioides* have been confirmed by site-directed mutagenesis (Cane et al., 1995a; Cane & Xue, 1996). The acidic aspartate-rich motif (I,L,V)XDDXXD, has been found in TS from all five sources as well as in other sesquiterpene, diterpene, and monoterpene synthases (Table 1). This aspartate-rich sequence was first noted in yeast farnesyl diphosphate synthase and in several other prenyl transferases as well (Ashby & Edwards, 1990; Marrero et al., 1992; Joly & Edwards, 1993). It has been proposed that the aspartate residues chelate the divalent Mg^{2+} ion that is required for substrate binding (Ashby & Edwards, 1990).

Beyond the presence of these two characteristic motifs, little is known about the active site of any sesquiterpene synthase. We describe experiments using site-directed mutagenesis to examine the role of the three aspartate residues in the trichodiene synthase reaction.

MATERIALS AND METHODS

Materials. Sources of reagents, recombinant trichodiene synthase from *E. coli* BL21(DE3)/pZW03 and chromatographic materials were as previously described (Cane et al., 1995a). [1-^{3}H]FPP (138.6 mCi/mol) was prepared by diluting stock purchased from NEN DuPont with synthetic FPP, prepared as described (Cane & Yang, 1994). λ DNA/*Hin*dIII, ΦX174/*Hae*III, *Pfu* DNA polymerase, *Nco*I, *Nsi*I, *Bg*/II, and *E. coli* XL1-Blue were purchased from Stratagene (San Diego, CA). The 1-kb and 100-bp DNA ladders were purchased from Gibco BRL (Gaithersburg, MD). *Sca*I was purchased from New England Biolabs (Beverly, MA). T4 DNA ligase and the Wizard Minipreps DNA Purification System were purchased from Promega (Madison, WI). *E.*

coli BL21(DE3) was purchased from Novagene (Madison, WI). Ultra-Free Probind filters units were purchased from Millipore (Milford, MA). DNA Sequence kit Version 2.0 was purchased from U.S. Biologicals (Cleveland, OH). Sephadex G-25 was from Pharmacia (Piscataway, NJ).

General Methods. General methods for spectroscopic analysis, protein purification and analysis, trichodiene synthase assay, and numerical analysis of kinetic data were as described (Cane et al., 1995b). Buffer T consisted of 10 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 15% glycerol, and 5 mM β-mercaptoethanol. For divalent metal ion dependence experiments with Mn^{2+}, all incubations and assays were performed in buffer T lacking $MgCl_2$ but containing 0.01 mM $MnCl_2$. PCR and ligation reactions were run in a Coy Laboratory Products model 50/60 TempCycler (Ann Arbor, MI). Restriction enzyme digestions were run at 37 °C in a Fisher Brand 10 mm dry bath. Preparative volume centrifugations were performed using a DuPont Sorvall RC5 centrifuge at 4 °C (Wilmington, DE). Mass spectra were obtained using an HP 5890 Series II Plus gas chromatograph interfaced to an HP 5988A quadrupole mass spectrometer with a Vectra 5/90c data system. NMR spectra were obtained on Bruker AM 400 spectrometers at 400.134 MHz for ^1H and 100 MHz for ^{13}C. $^1H-^1H$ COSY, HMQC, and HMBC spectra were obtained on a Varian Unity-500 spectrometer (operating at 499.84 MHz for ^1H and 125.7 MHz for ^{13}C). All analytical TLC plates were visualized by iodine or by spraying with 4% *p*-anisaldehyde in 95% ethanol containing 2% H_2SO_4. Steady state kinetic parameters were initially estimated from Lineweaver-Burk double-reciprocal plots and then calculated by direct fitting of data to the Michaelis−Menten equation using nonlinear least-squares methods.

Preparation of Plasmid pZW03. *E. coli* XL1-Blue/pZW03 cells were inoculated into 5 mL of LBA media and incubated at 37 °C on a shaker (300 rpm) overnight. Cells were pelleted from 3 mL of culture by microcentrifugation for 2 min at 12 000*g*. The cells were resuspended in 200 μL of Cell Resuspension Solution, and DNA minipreps were performed following the protocols from the Wizard Minipreps DNA Purification Technical Bulletin to give 35 μg of pZW03 DNA, as determined by 1% agarose gel electrophoresis using 150 ng of λDNA/*Hin*dIII and 150 ng of 1-kb ladder as standards.

Plasmids for Site-Directed Mutagenesis of Amino Acids 100, 101, and 104. Asp residues 100, 101, and 104 were each replaced by Glu by PCR mutagenesis. The trichodiene synthase expression vector, pZW03, was used as the template for PCR. The 25-mer forward primer incorporated a *Bg*/II restriction site (underlined) by a silent point mutation which altered the GCC alanine codon to GCA (boldface) (5′-C TCT AAA GAA TGC ATG **GCA** GAT CTA-3′). The three 48-mer reverse primers each incorporated a point mutation which altered a codon from aspartate (GAC or GAT) to glutamate (boldface): (Asp100 to Glu100) 5′-ATA GTT TAC CAT GGT TGG GTA CGG GTC ATC CTT GCT ATC **CTC** CAA AAC-3′; (Asp101 to Glu101) 5′-ATA GTT TAC CAT GGT TGG GTA CGG GTC ATC CTT GCT **TTC** GTC CAA AAC-3′; (Asp104 to Glu104) 5′-ATA GTT TAC CAT GGT TGG GTA CGG GTC **TTC** CTT GCT ATC GTC CAA AAC-3′. The optimal reaction mixture for PCR amplification of the mutagenic insert was as follows: 20 pmol of DNA primers, 10 ng of pZW03 template, 2.5 mM dNTPs, 350 μM dNTPs, 2 mM $MgCl_2$, and a 10× dilution of Stratagene P*fu* DNA polymerase reaction buffer 2. Following an initial 5-min incubation at 95 °C, the reaction mixtures were transferred to an ice bath; 3 units of P*fu* polymerase was added to each tube, and the reaction mixtures were returned to the thermal cycler for 3 min at 65 °C. Thirty-five cycles, each consisting of a 3-min extension at 76 °C, a 2-min denaturation at 95 °C, and a 2-min annealing at 65 °C, were performed. The reaction program concluded with a final 7-min extension at 76 °C before holding at 4 °C until the reaction vials were removed from the cycler. Passage through a Probind filter by centrifugation at 12 000*g* for 30 s, ethanol precipitation with 2.5 vol of ice-cold ethanol, and centrifugation at 12 000*g* for 30 min yielded a total of 360 ng of a 100-bp PCR-amplified product as determined by 4% agarose gel electrophoresis using 150 ng of ΦX174/*Hae*III and 150 ng of 100-bp ladder as standards.

Preparation of the Overexpression Plasmids pBF01, pQX01, and pQX02. Each PCR product and the pZW03 vector were individually digested with *Nco*I and *Nsi*I by incubating 1.6 μg of PCR-amplified product or 6 μg of plasmid DNA with 3 μL (30 units of *Nco*I) or 20 μL (40 units of *Nsi*I) of endonuclease in 4 or 8 μL Stratagene 10× universal buffer. Reaction mixtures were incubated for 5−9 h at 37 °C, and complete digestion was monitored by agarose gel electrophoresis. Reaction mixtures were passed through a Probind filter by centrifugation at 12 000*g* for 30 s. DNA digestions were purified by ethanol precipitation in the presence of 0.3 M of sodium acetate. Following determination of the DNA concentration by agarose gel electrophoresis, the PCR amplified insert and the pZW03 vector were ligated. The ligation mixture contained 200 ng of pZW03/*Nco*I/*Nsi*I (4 μL of 50 ng/μL stock), 34 ng of insert/*Nco*I/*Nsi*I (1 μL of 34 ng/μL stock), and 2 μL of H_2O. This mixture was heated to 65 °C for 5 min and then chilled on ice before the addition of 2 μL of 5× Promega T4 DNA ligase reaction buffer, 1 μL of ATP (0.01 M, pH = 7) and 1 μL of T4 DNA ligase (1 unit/μL). The reaction mixture was incubated for 17 h at 14 °C. This crude reaction mixture was used directly for the transformation of *E. coli* XL1-Blue.

Transformation of E. coli XL1-Blue and BL21(DE3). Transformation of competent *E. coli* cells employing selection for ampicillin resistance was performed following published procedures (Sambrook et al., 1989). Plasmids isolated from *E. coli* XL1-Blue/pBF01, -pQX01, and -pQX02, were used to transform the expression host *E. coli* BL21-(DE3). Miniprep isolation gave plasmids pBF01, pQX01, and pQX02, which were analyzed by agarose gel electrophoresis to confirm their size and digested with *Bgl*II to confirm the presence of the PCR-amplified mutagenic insert.

Overexpression and Purification of Mutant Trichodiene Synthases. *E. coli* BL21(DE3)/pBF01, -pQX01, and -pQX02 were each used to inoculate 100 mL of LBA media. *E. coli* BL21(DE3)/pZW03 (wild type) was also inoculated as a control. The cultures were incubated at 30 °C on a shaker (250 rpm) until an OD_{600} of 0.7−0.8 was reached. IPTG (1 M, 0.1 mL) was added to each culture to a final concentration of 1 mM to induce synthesis of TS, and the cultures were incubated at 30 °C on a shaker (250 rpm) for 3.5 h. The production of trichodiene synthase was monitored by SDS−PAGE every half-hour after addition of IPTG. Mutant trichodiene synthases, overproduced at the same level as for

the wild type, were purified to homogeneity based on published procedures (Cane et al., 1995b). Exchange from buffer T to buffer containing 0.01 mM of $MnCl_2$ employed a Sephadex G-25 (Fine) column.

DNA Sequencing. DNA sequencing of both strands of the mutagenized inserts in the TS coding sequence for each mutant plasmid was performed with the Sequenase kit version 2.0 on double-stranded templates employing the dideoxy chain termination method (Sanger et al., 1977). Sequencing reactions were labeled with [α-^{35}S]ATP. Sequence analysis employed the GCG software package (Genetics Computer Group, Madison, WI).

Characterization of Products from Trichodiene Synthase Mutants by GC−MS. In a typical experiment, either crude cell lysate or purified protein from *E. coli* BL21(DE3)/pBF01 (D100E) (100 μL) was incubated with 162 μM FPP in 1 mL buffer T at 30 °C for 2 h. The hydrocarbon product was extracted with HPLC-grade pentane and purified on a silica gel column (3 cm) packed in a Pasteur pipette overlaid with sodium sulfate (0.5 cm). Identical incubations were performed with the crude cell lysate and purified protein from *E. coli* BL21(DE3)/pZW03 (wild type trichodiene synthase). The extract of each incubation was concentrated under reduced pressure at 0 °C, and the concentrates (1−3) μL were analyzed by GC−MS. The observed mass spectra were compared with that of authentic trichodiene and other reference sesquiterpene standards. Analogous experiments were also carried out with the D101E and D104E mutants.

Characterization of Products from Trichodiene Synthase Mutants D100E by NMR. Preparative scale incubation of crude TS D100E (15 μmol) and FPP (0.22 mmol) was carried out in 2 L of Tris buffer (10 mM Tris-HCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, pH = 7.8) at 30 °C overnight. The hydrocarbon products were extracted with HPLC-grade pentane. The pentane extract was passed through a silica gel column overlaid with anhydrous sodium sulfate, and the eluate was concentrated at 0 °C under reduced pressure to yield 11 mg of crude product (overall yield, 25%). The mixture of hydrocarbons was partially purified by column chromatography (SiO_2, pentane) and further purified by argentation chromatography (SiO_2/$AgNO_3$, pentane/ethyl ether). Silver nitrate-impregnated silica gel was prepared in darkness as follows: $AgNO_3$ (Aldrich, 25 g) was dissolved in a 1:1 (v/v) mixture of CH_3CN (125 mL) and ethanol (125 mL). Silica gel 60 (100 g) was added to the solution. The mixture was stirred before the solvent was removed *in vacuo* over a 20−60 °C temperature range. The silica gel was dried under high vacuum at 80 °C for several hours and activated in an oven (150 °C) overnight. Silver nitrate-impregnated TLC plates were prepared by dipping analytical TLC plates into the same solution as above and were dried in an oven (150 °C) overnight. Each of the known sesquiterpene products was identified by detailed comparison of their spectra with published spectra or with authentic reference samples. The details of the structure assignment of isochamigrene, based on ^{1}H and ^{13}C NMR, combined with INEPT, NOE, ^{1}H−^{1}H COSY, HMQC, and HMBC experiments, are described elsewhere (Cane et al., 1996).

RESULTS

Characterization of D100E, D101E, and D104E mutants. To investigate the functional roles of Asp100, Asp101, and Asp104 in catalysis by trichodiene synthase, mutant strains of *E. coli* BL21(DE3) carrying pBF01 (D100E), pQX01 (D101E), and pQX02 (D104E) were constructed by site-directed mutagenesis (Table 2). Each TS mutant was purified to homogeneity, and the steady state kinetic parameters, K_m and k_{cat}, were determined (Table 3).

Table 2: Site-Directed Mutations in Trichodiene Synthase

plasmid	Asp−Glu mutations (amino acids 98−102)[a]
pZW03	GAC GAT AGC AAG GAT
pBF01	GA**G** GAT AGC AAG GAT
pQX01	GAC GA**A** AGC AAG GAT
pQX02	GAC GAT AGC AAG GA**A**

[a] Nucleotide substitutions are indicated in bold with underlining.

The k_{cat} for the D100E mutant was reduced only 2.5-fold relative to that of the wild type enzyme, whereas the K_m was increased 8-fold. Similarly, the k_{cat} of D101E was reduced 3.5-fold whereas the K_m was increased 1.5-fold. On the other hand, both k_{cat} and K_m of D104E remained unchanged.

Characterization of Sesquiterpene Hydrocarbons Produced by the D100E Mutant. Incubation of TS D100E with FPP gave rise to a mixture of trichodiene and five additional sesquiterpene hydrocarbons, each *m/z* 204, as revealed by GC−MS analysis. Chromatographic purification of the crude pentane extract of a preparative incubation yielded 3.7 mg of trichodiene (**2**), 0.9 mg of β-farnesene (**5**), 0.8 mg of (−)-(*Z*)-α-bisabolene (**6**), 0.9 mg of β-bisabolene (**7**), 0.8 mg of cuprenene (**8**), and 0.8 mg of (**9**). The individual sesquiterpene hydrocarbons were identified by comparison of GC−MS retention times and mass fragmentation patterns with known standards as well as by detailed comparison with authentic NMR spectra (Delay & Ohloff, 1979; Miyazawa & Kameoka, 1983; Dauben & Oberhänsli, 1966; Makayama et al., 1975; Tkachev et al., 1991; Cane & Xue, 1996). A combination of ^{1}H and ^{13}C NMR with ^{1}H−^{1}H COSY, INEPT, NOE, HMQC, and HMBC spectra (Cane et al., 1996) led to the assignment of structure **9**, named as isochamigrene, corresponding to a previously unidentified sesquiterpene carbon skeleton [cf. chamigrene, Tanaka et al. (1968)].

Metal-Ion Dependence of Trichodiene Synthase Mutants. To probe further the role of the aspartate-rich domain in trichodiene synthase in chelating the divalent metal ion, we examined the differential effects of replacing Mg^{2+} by Mn^{2+} in incubations with each mutant. Since high concentrations of manganese ion inhibit trichodiene synthase (Hohn & VanMiddlesworth, 1986), a concentration of 0.01 mM of $MnCl_2$ was used. The steady state kinetic parameters, K_m and k_{cat}, were determined for each mutant (Table 3) and compared with those measured in the presence of 5 mM $MgCl_2$ (Table 3, Figure 1). Substitution of Mn^{2+} for Mg^{2+} had negligible effect on the K_m for FPP, but reduced the k_{cat} by a factor of about 4. A similar effect was observed for the D104E mutant. On the other hand, while both the D100E and D101E mutants showed changes in k_{cat} and K_m in the presence of Mn^{2+}, the relative k_{cat}/K_m for each of the latter

Table 3: Kinetic Parameters for Wild-Type and Mutant Trichodiene Synthase

	Mg^{2+} [a]			Mn^{2+} [b]		
trichodiene synthase	K_m (nM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})	K_m (nM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
wild-type	78.0 ± 5.6	0.138 ± 0.004	1.77×10^6	84.8 ± 11.2	0.0357 ± 0.002	4.21×10^5
D100E	657.3 ± 72.0	0.053 ± 0.003	8.06×10^4	218.8 ± 27.6	0.0192 ± 0.0007	8.78×10^4
D101E	123.8 ± 3.5	0.040 ± 0.0002	3.21×10^5	12.9 ± 0.76	0.00246 ± 0.00002	1.91×10^5
D104E	72.9 ± 5.6	0.133 ± 0.005	1.82×10^6	69.6 ± 5.89	0.047 ± 0.001	6.75×10^5

[a] Buffer T. [b] 10 mM Tris-HCl (pH 7.8), 0.01 mM $MnCl_2$, 15% glycerol, 5 mM β-mercaptoethanol.

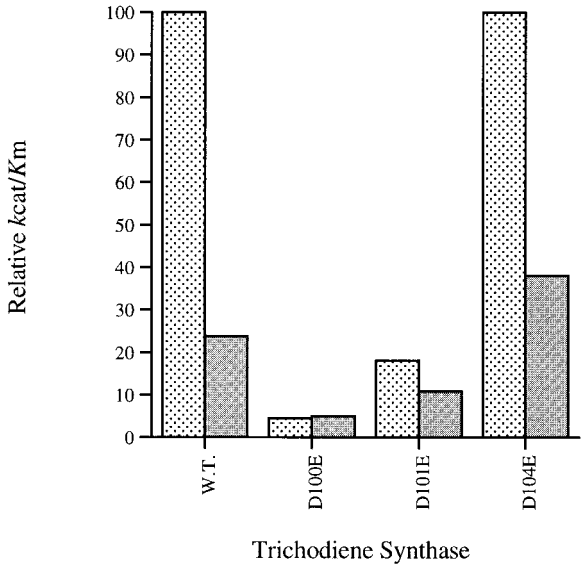


FIGURE 1: Comparison of wild type and mutant trichodiene synthase activities (k_{cat}/K_m) with Mg^{2+} (left bars) and with Mn^{2+} (right bars).

Table 4: Relative Proportion of Sesquiterpenes Produced by Mutant Trichodiene Synthases[a]

	D100E		D101E		D104E	
	Mg^{2+} [b]	n^{2+} [c]	Mg^{2+} [b]	Mn^{2+} [c]	Mg^{2+} [b]	Mn^{2+} [c]
cuprenene (**8**)	7	17	3	13	3	5
isochamigrene (**9**)	14	26	9	13	4	4
2, **5**, **6**, **7**[d]	79	57	88	74	93	91

[a] Measured by GC−MS as described in general methods. [b] Buffer T. [c] 10 mM Tris-HCl (pH 7.8), 0.01 mM $MnCl_2$, 15% glycerol, 5 mM β-mercaptoethanol. [d] Under the GC conditions used for this series of analyses, **2**, **5**, **6**, and **7** were not resolved. The relative proportions of the four compounds could be determined in selected cases by ^{1}H NMR. For the D100E mutant, **2**, **5**, **6**, and **7** were isolated in a ratio of ca. 4:1:1:1.

two mutants was essentially unaffected by the metal ion substitution. A more significant effect was observed on the distribution of anomalous cyclization products. Although wild type TS produced exclusively trichodiene in the presence of either divalent metal ion, replacement of Mg^{2+} by Mn^{2+} increased the proportion of cuprenene (**8**) and isochamigrene (**9**) produced by each mutant, as determined by GC−MS (Table 4).

DISCUSSION

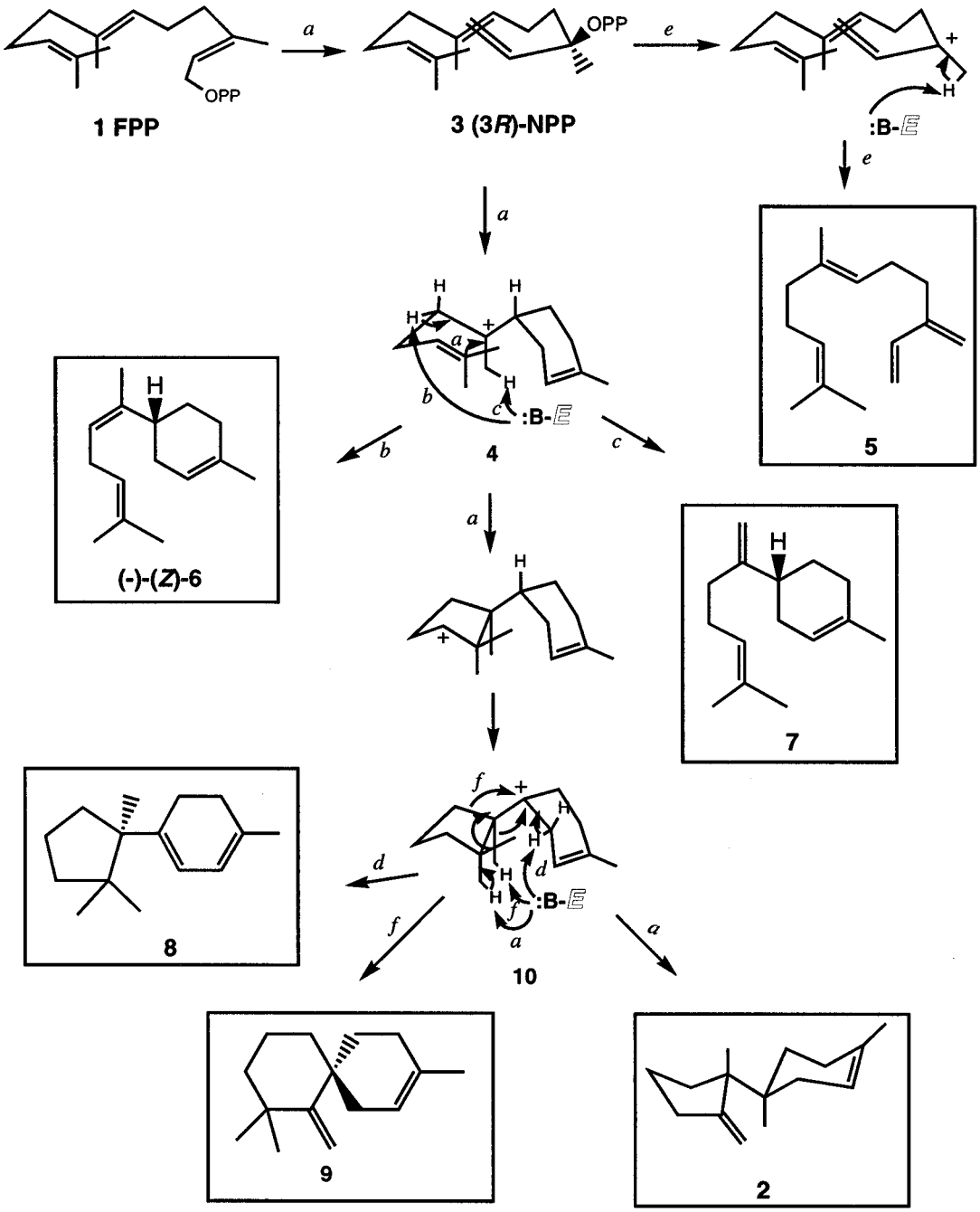
Site-Directed Mutagenesis on the Aspartate-Rich Domain in Trichodiene Synthase. On the basis of the presence of two (I,L,V)XDDXXD motifs in prenyltransferases, Edwards originally postulated that each aspartate-rich domain might be responsible for chelation of a divalent magnesium cation, each of which would complex with an individual isopentenyl diphosphate or allylic diphosphate substrate (Ashby & Edwards, 1990). This suggestion has subsequently been supported by site-directed mutagenesis (Marrero et al., 1992; Joly & Edwards, 1993; Song & Poulter, 1994) which showed significant decreases in k_{cat} for both yeast and rat farnesyl diphosphate synthases as a consequence of replacement of individual Asp residues with Glu or Ala. In some cases, replacement of the third Asp residue in one of the domains by Glu had little effect on k_{cat}. Further direct evidence for the role of two of the three aspartates in each domain in chelating the divalent cation has come from crystallographic analysis of avian FPP synthase (Tarshis et al., 1994).[2] It is therefore of great interest that in spite of the lack of significant sequence similarity between microbial sesquiterpene synthases and any other known protein, the same aspartate-rich motif is found in all of these Mg^{2+}-dependent cyclases for which FPP is the substrate (Cane, 1990)[3] (Table 1). The study of the aspartate-rich domain of trichodiene synthase reported here has established that replacement of either Asp100 or Asp101 with Glu results in a modest increase in the observed K_m combined with a small decrease in k_{cat}, resulting in a net 5−20-fold decrease in k_{cat}/K_m. Replacement of Asp104 with Glu, on the other hand had negligible effect on either of the observed steady state kinetic parameters.

Enzymatic Formation of Multiple Sesquiterpene Hydrocarbons. Although there are more than 200 distinct sesquiterpene carbon skeletons already known, each of these metabolites can be derived by electrophilic cyclization of the universal acyclic precursor FPP. Remarkably, each member of this family of enzymes, all using the same substrate and variations on the same fundamental cyclization mechanism, is able to generate its characteristic product with exquisite specificity and high fidelity. A major determinant of the structure and stereochemistry of the eventually formed product appears to be the precise folding of the substrate FPP at the cyclase active site, as well as the accurate control of the timing and site of quenching of the positive charge.

[2] More recently, Poulter and his collaborators have obtained crystal structures on FPP synthase mutants showing Mg^{2+} chelated to the nonbridging oxygens of the pyrophosphate moiety of an allylic substrate as well as to two adjacent Asp residues in one of the aspartate-rich domains (personal communication, C.D. Poulter).

[3] A search of the PIR database revealed the presence of a large number of proteins with a similar aspartate-rich motif, including numerous kinases and pyrophosphokinases, DNA and RNA polymerases, amino acid-tRNA ligases, and NAD protein ribosyl transferases, many of which catalyze the Mg^{2+}-dependent displacement of a pyrophosphate, adenosyl diphosphate, or adenylate moiety. On the other hand, Mg^{2+} chelation cannot be the only role for this aspartate-rich sequence, since the same motif can also be found in a variety of other proteins of diverse function, including calmodulin, cytochrome P450s, triosephosphate isomerase, and β-lactamase.

Scheme 2: Enzymatic Formation of Multiple Sesquiterpenes from FPP



We have previously shown that mutants of trichodiene synthase altered in a base-rich domain, believed to be involved in binding of the pyrophosphate moiety, produce mixtures of sesquiterpene hydrocarbons, including (−)-(*Z*)-α-bisabolene (**6**), *β*-bisabolene (**7**), and cuprenene (**8**) (Cane & Xue, 1996). We have previously proposed that binding of FPP to the site-directed mutants results in small but important changes in the precise positioning and folding of the substrate within the active site. When the aberrantly bound FPP undergoes ionization, abortive cyclization products can be generated by premature deprotonation of the normal cationic cyclization intermediates, possibly by the same active site base that is responsible for the final deprotonation step in trichodiene biosynthesis. The products thus formed not only reveal the role played by the various residues at the cyclase active site, but provide strong evidence for the structures of the various cationic intermediates which normally are completely sequestered by the wild type trichodiene synthase. The formation of **5**−**8** by each of the three trichodiene synthase Asp→Glu mutants, is fully consistent with this same model, illustrated in Scheme 2, pathways b−e. At the same time, the formation of multiple products by these mutants establishes the importance of the aspartate-rich domain in catalysis of the trichodiene synthase reaction. Notably, even the D104E mutant produces small amounts of anomalous cyclization products, in spite of the fact that its overall steady state kinetic parameters are indistinguishable from those of wild type trichodiene synthase. The formation of isochamigrene is also noteworthy. The latter compound cannot be generated directly by deprotonation of one of the normal cyclization intermediates. Instead, the cuparenyl cation **10** must undergo an alternative ring expansion and deprotonation (pathway f), presumably resulting from the loss of precise control consequent on the

aberrant binding of the FPP substrate and its derived cationic cyclization intermediates. Interestingly, isochamigrene itself has never been reported in nature and can therefore be considered one of a newly emerging class of "unnatural" natural products.[4]

Role of the Divalent Metal Ion in Catalysis. Trichodiene synthase, like all other sesquiterpene and related monoterpene and diterpene synthases, has an absolute requirement for a divalent metal ion, with Mg^{2+} being preferred. Previous studies have shown that low concentrations of Mn^{2+} can substitute for Mg^{2+}, while higher concentrations are strongly inhibitory (Hohn & VanMiddlesworth, 1986). The divalent metal presumably plays a role both in substrate binding and in catalysis. Chelation of Mg^{2+} neutralizes two of the three negative charges of the pyrophosphate moiety of FPP, thereby assisting in the acid-catalyzed ionization of the allylic substrate. Indeed, Mg^{2+} itself can catalyze the solvolysis of FPP at pH 7 in the absence of enzyme (Chayet et al., 1984). Chelation of the Mg^{2+} ion, bound to FPP, by two of the active site Asp residues can properly position the substrate in the active site in preparation for initiation of cyclization by ionization of the allylic diphosphate substrate. In order to probe further the interaction of the conserved aspartate residues and the divalent metal, we examined the effect of replacing Mg^{2+} with Mn^{2+} both on steady kinetic parameters and on the distribution of anomalous cyclization products in the three Asp→Glu mutants, using wild type trichodiene synthase as a control.[5] In principle there should be only relatively minor differences in the geometry of interaction of each divalent metal ion with the carboxylate anion moieties of the aspartate residues (da Silva & Williams, 1991). Given the proposed sensitivity of the cyclization reaction to subtle changes in substrate binding geometry, however, it was anticipated that these small differences might have a significant effect on the rate or specificity of the trichodiene synthase reaction. In fact, substitution of Mn^{2+} for Mg^{2+} decreased the observed k_{cat}/K_m for both the wild−type and the D104E mutants by factors of 4 and 2.5, respectively. Interestingly, while substitution of Mg^{2+} by Mn^{2+} lowered K_m and k_{cat} for both the D100E and D101E mutants, the relative k_{cat}/K_m values of both the D100E and D101E mutants were effectively insensitive to this substitution (Table 3 and Figure 1). Mn^{2+} also exerted a pronounced effect on the proportion of aberrant sesquiterpenes generated by each mutant. Thus although wild type trichodiene synthase produced exclusively trichodiene in the presence of either metal ion, use of Mn^{2+} approximately doubled the relative proportions of both cuprenene (**8**) and isochamigrene (**9**) for both the D100E and D101E mutants (Table 4). A smaller, but still significant (ca 50%), increase in the proportion of **8** resulted from incubation of the D104E mutant with Mn^{2+} in place of Mg^{2+}. Together these results suggest that Asp100 and Asp101 play more important roles in catalysis than does Asp104. Studies involving binding of substrate analogs to wild type and mutant trichodiene synthases as well as growth of protein crystals suitable for crystallographic analysis are in progress.

[4] For other recent examples of the formation of novel metabolites by site-directed modification of enzymes of natural product biosynthesis, cf. the following: Ohnuma, S., Nakazawa, T., Hemmi, H., Hallberg, A.-M., Koyama, T., Ogura, K., & Nishino, T. (1996) *J. Biol. Chem. 271*, 10087−10095; Back, K., & Chappell, J. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 6841−6845; Pieper, R., Luo, G., Cane, D. E., & Khosla, C. (1995) *J. Am. Chem. Soc. 117*, 11373−11374.

[5] For a recent example of the alteration of the kinetics of a cAMP-dependent protein kinase by substitution of Mn^{2+} for Mg^{2+}, see B. D. Grant and J. A. Adams [(1996) *Biochemistry 35*, 2022−2029].

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