Pentalenene Synthase. Histidine-309 Is Not Required for Catalytic Activity

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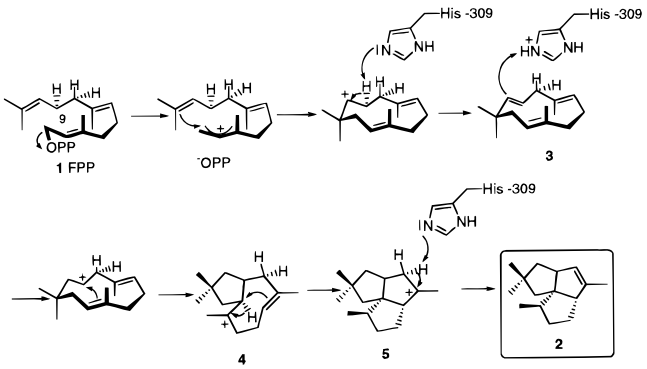
Received October 19, 1998

Sesquiterpene synthases are ubiquitously represented in marine and terrestrial plants, fungi, and certain microorganisms. Together these enzymes are capable of converting the universal acyclic precursor, farnesyl diphosphate (FPP, **1**), into more than 300 distinct sesquiterpenes.[1] The molecular diversity in product structure and stereochemistry is, to a great extent, controlled by the precise folding of the FPP substrate at the cyclase active site. After being properly folded, FPP undergoes an ionization followed by a precise sequence of intramolecular electrophilic addition reactions and cationic reactions, including rearrangements, terminated by a final deprotonation or capture of an external nucleophile such as water.[2] Very little is known, however, about the way terpene synthases catalyze and control these intricate cyclizations.

Pentalenene synthase (PS) catalyzes the conversion of FPP (**1**) to pentalenene (**2**), the hydrocarbon precursor of the pentalenolactone family of antibiotics.[3] The cyclase from *Streptomyces* UC 5319, a monomer of 38 kDa, has been cloned and overexpressed in *Escherichia coli*.[3b] Extensive studies support the cyclization mechanism, illustrated in Scheme 1, in which FPP is converted to **5** by way of humulene (**3**) and the seco-illudyl cation **4**. Deprotonation of **5** would give pentalenene (**2**).[3a,c−e] We had postulated that a single active-site base might be responsible for the successive deprotonation−reprotonation−deprotonation steps.[3a] Indeed, the X-ray crystal structure of recombinant *Streptomyces* UC 5319 pentalenene synthase (EC 4.6.1.5)[4] revealed that histidine-309 (H309) appeared to be ideally situated for abstraction of the relevant protons from FPP and derived intermediates.

To investigate the proposed role of H309 as the active-site base, we have constructed a set of PS mutants in which this histidine has been replaced by alanine, serine, cysteine, or phenylalanine. The individual plasmids pGZ11 (H309A), pGZ12 (H309S), pGZ13 (H309C), and pMS11 (H309F) were constructed by site-directed mutagenesis[5] (Table 1). Each of the resulting PS mutants was purified to homogeneity and the steady-state kinetic parameters were determined as described for wild-type recombinant pentalenene synthase.[3b] Unexpectedly, all four mutants retained substantial cyclase activity, with only minor increases in K_m for three of the mutants and decreases in k_{cat} ranging from as little as 3-fold for H309A and H309S to at most ca. 17-fold for the H309F mutant, with consequently relatively minor changes in k_{cat}/K_m for all but the H309F mutant.

Scheme 1



The products generated by each of the four histidine mutants were also analyzed by GC−MS, revealing that the major product pentalenene (**2**) was accompanied in each case by as much as 20% of varying proportions of two additional sesquiterpene hydrocarbons (m/z = 204) **6** and **7** (Table 1). Chromatographic purification (SiO_2 plates, pentane) of the crude pentane extract of a preparative scale incubation of FPP with mutant pentalenene synthase H309A gave two major fractions, I (R_f = 0.8) containing pentalenene (**2**) plus **6**, and II (R_f = 0.52) corresponding to **7** (0.3 mg).[6,7] Argentation chromatographic purification of fraction I ($SiO_2/AgNO_3$, CH_2Cl_2) gave 5 mg of pentalenene (**2**) (R_f = 0.63) and 0.6 mg of **6** (R_f = 0.21).

The 400 MHz ^{1}H NMR spectrum of **6** revealed the presence of three methyl groups attached to quaternary carbon atoms (0.93, 1.04, and 1.05 ppm) and one allylic methyl (1.56 ppm) as well as the absence of olefinic protons. The 100.61 MHz ^{13}C NMR spectrum displayed 13 signals between 15 and 50 ppm. We therefore deduced that **6** contained three rings and a fully substituted double bond. Detailed analysis of the ^{1}H−^{1}H COSY, HMQC, and HMBC spectra led to the assignment of **6** as Δ^6-protoilludene (Scheme 2), completely consistent with the reported NMR data for the sesquiterpene hydrocarbon previously isolated from the mycelial extract of the fomannosin producer, *Fomitopsis insularis*.[8]

The identification of **7** was more demanding due to the instability of this sesquiterpene which underwent conversion in $CDCl_3$ to a mixture of two additional sesquiterpene hydrocarbons, **8** (80%) and **9** (20%), each m/z = 204, as revealed by GC−MS. The 400 MHz ^{1}H NMR spectrum of this mixture of **8** and **9** revealed the presence in **8** of four olefinic protons (δ 4.72−4.70 and 4.42), a methyl singlet (δ 0.73), and an allylic methyl (δ 1.75). The HMQC spectrum of the mixture showed a correlation between two of these olefinic protons (δ 4.43 and 4.71) with the

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(5) Plasmid pZW05[3b] was first double-digested with *Eco*RI and *Hin*dIII and ligated into the corresponding sites of the pALTER-1 vector obtained from Promega. The resulting plasmid was mutated using the Altered Sites II in vitro Mutagenesis systems from Promega. The mutagenic plasmid was analyzed by sequencing to confirm the mutation before being double-digested with *Eco*RI and *Hin*dIII and ligated back into the T7-based expression plasmid pLM1.

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(7) In a typical preparative scale incubation, FPP (**1**) (50 mg, 0.1 mmol), prepared as previously described,[6] was incubated with the H309A mutant of recombinant *Streptomyces* UC 5319 pentalenene synthase (0.5 μM) from *E. coli* BL21(DE3)/pGZ11 (H309A) in 0.5 L of buffer (50 mM Tris-HCl, 20% (v/v) glycerol, 5 mM $MgCl_2$, 0.03 mM $MnCl_2$, 0.2 mM benzamidine, 0.2 mM PMSF, and 1 mM DTT, pH = 8.2) for 16 h at 30 °C. The mixture was then extracted with pentane, and the combined pentane extracts were concentrated to 10 mL under vacuum at 0 °C and applied to a 2-cm silica gel column overlaid with $MgSO_4$ to remove water.
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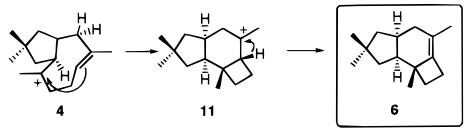
10.1021/ja983657h CCC: $18.00
Published on Web 01/08/1999

Table 1. Pentalenene Synthase Mutants

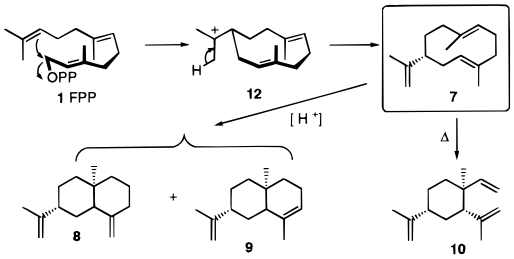
plasmid	enzyme	nucleotide sequence[a]	steady-state kinetic parameters			relative proportion of sesquiterpenes (%)		
			k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} M^{-1})	**2**	**6**	**7**
pZW05	WT PS	TAC GAC TGG CAC CGG TCC TCG	0.3	0.3	1.0×10^6	100	0	0
pGZ11	H309A	TAC GAC TGG **GC**C CGG TCC TCG	0.11	0.19	0.6×10^6	78	12	10
pGZ12	H309S	TAC GAC TGG **AG**C CGG TCC TCG	0.11	0.25	0.4×10^6	82	8	10
pGZ13	H309C	TAC GAC TGG **TG**C CGG TCC TCG	0.07	0.33	0.2×10^6	80	7	13
PMS11	H309F	TAC GAC TGG **TT**C CGG TCC TCG	0.018	1.43	1.2×10^4	84	3	13

[a] Nucleotide substitutions are indicated in bold.

Scheme 2



Scheme 3



^{13}C NMR signal at 105.3 ppm and also a correlation between the other two protons (δ 4.70 and 4.72) and the olefinic ^{13}C resonance at 108.12 ppm, implying that **8** was a bicyclic sesquiterpene with two exomethylene double bonds, one of which carried an attached methyl group. Moreover the HMBC spectrum of the mixture of **8** and **9** displayed a correlation of the methyl at δ 0.73 with four carbon signals at 35.96, 41.16, 41.96, and 49.88 ppm, suggesting that **8** was β-selinene. This conclusion was firmly established by direct comparison of the ^{1}H NMR, ^{13}C NMR, HMQC, HMBC, and GC−MS spectra of the mixture of **8** and **9** with those of an authentic sample of β-selinene extracted from celery seeds.[9] GC−MS examination of the same celery seed extract also led to the identification of **9** as α-selinene.

Germacrene A is known to be particularly susceptible to proton-induced cyclizations to give β-selinene (**8**) and α-selinene (**9**)[10,11] and to undergo facile thermal Cope rearrangement to β-elemene (**10**). (Scheme 2) The identity of **7** as germacrene A was confirmed by direct comparison of the GC−MS spectra of **7** with an authentic sample of germacrene A,[12] a conclusion which was further supported by thermal rearrangement of the enzymatically derived **7** to β-elemene (**10**) upon GC−MS analysis using an injection port temperature of 250 °C.[11]

These results establish conclusively that H309 in pentalenene synthase is not required for cyclase activity and would appear to rule out a role for this amino acid residue as the active-site base. Although the proposed interconversion of the two intermediate humulyl cations may well take place by a 1,2-hydride shift in place of the previously proposed deprotonation−reprotonation sequence (in the wild-type as well as in the mutant synthases), removal of a proton derived from H-8 of FPP is still required for eventual formation of pentalenene.[13] Since the penultimate carbocationic intermediate **5** would presumably have a pK_a in the range of −10, the presumptive active-site base hardly needs to be very basic, yet no other basic amino acid residues appear to be properly positioned to mediate this deprotonation. On the basis of these experiments, we cannot as yet rule out the possibility that H309 serves as the natural base in a step that is rapid compared to the rate-determining catalytic steps but that some other group might serve as a surrogate base in the mutant PS enzymes. One intriguing possibility is that the carbonyl oxygen atom of the side-chain amide of Asn-219 or of a peptide moiety in the protein backbone itself might serve as this surrogate or even the natural base for the formation of **2**.[14]

The formation of Δ^6-protoilludene **6** by the four H309 mutants is readily explained by direct cyclization of the natural seco-illudyl cation intermediate **4** (Scheme 2) followed by deprotonation of the resulting tricyclic carbocationic species **11**. (Scheme 3) By contrast, formation of germacrene A requires an alternate Markovnikov addition to the 10,11-double bond of FPP and deprotonation of one of the two attached methyl groups in **12**. (Scheme 3) The two anomalous cyclizations are presumably made possible by the relaxed control over substrate and intermediate conformations in the active sites of the various H309 mutants.[15] Notably, the deprotonations leading to the formation of both **6** and **7** involve protons chemically and geometrically distinct from that lost in the generation of pentalenene, indicating that multiple amino acid residues (or peptide bonds) could be acting as adventitious active-site bases in the mutant cyclases.

Acknowledgment. We thank Dr. Maurice C. R. Franssen and Dr. Jan-Willem de Kraker (Wageningen Agricultural University, The Netherlands) for the gift of germacrene A, Dr. Edmund Graziani for advice and assistance in acquisition of the 2D NMR spectra, and Professor Rodney Croteau of the Institute for Biological Chemistry, Washington State University, for helpful discussions. This research was supported by an NIH Merit Award to D.E.C. (GM30301). K.U. was supported by the Brown University−Keio University Exchange Scholars Program.

Supporting Information Available: 400 MHz ^{1}H NMR spectra and tabulated ^{1}H and ^{13}C NMR data for **6** and **7**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) Germacrene A was a gift from Dr. Jan-Willem de Kraker (Wageningen Agricultural University, The Netherlands).

(13) Although in wild-type PS the proton which is removed from **5** to generate **2** corresponds to H-8*si* of FPP,[3d] the stereochemical course of the reactions catalyzed by the various histidine mutants has not yet been determined.

(14) Chappell and Noel have suggested that an active-site tryptophan (W273) can serve as the base in the mechanistically closely related cyclization catalyzed by epi-aristolochene synthase, a protein closely related in three-dimensional structure but with no similarity in sequence to pentalenene synthase. Indeed, these researchers have found that replacement of W273 abolishes cyclase activity in epi-aristolochene synthase. (Starks, C. M.; Back, K.; Chappell, J.; Noel, J. P. *Science* **1997**, *277*, 1815−1820.) Interestingly the latter cyclization is believed to involve germacrene A (**7**) as a mandatory intermediate. By contrast, we have observed that the corresponding PS double mutant, H309F/W308F, retains substantial cyclase activity (M. Seemann, unpublished observations.)

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