Trichodiene Synthase. Enzymatic Formation of Multiple Sesquiterpenes by Alteration of the Cyclase Active Site

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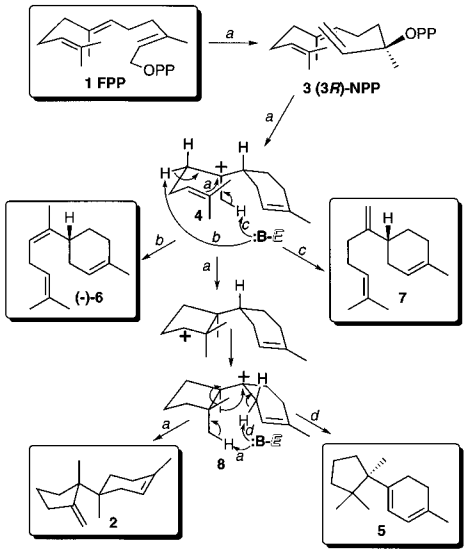
Sesquiterpene synthases are versatile catalysts which together are responsible for the formation of more than 200 distinct sesquiterpene carbon skeletons.[1] Intriguingly, each individual synthase is capable of converting the universal acyclic precursor farnesyl diphosphate (FPP, **1**) to a distinct sesquiterpene, all the while utilizing a common mechanism involving ionization of the allylic pyrophosphate ester followed by a precise sequence of intramolecular electrophilic addition reactions.[2] A major determinant of the structure and stereochemistry of the ultimately formed sesquiterpene is believed to be the precise folding of the FPP substrate at the cyclase active site. Although many of the mechanistic and stereochemical features of this model have been verified by a wealth of isotopic labeling experiments,[2,3] little is known about the active site of any cyclase or the manner in which a sesquiterpene synthase imposes a particular conformation on its highly lipophilic substrate, precisely controls the resulting cascade of electrophilic cyclizations, and ultimately quenches the positive charge.

Trichodiene synthase, an enzyme isolated from a variety of fungal sources,[4] catalyzes the conversion of FPP to trichodiene (**2**), the parent hydrocarbon of the trichothecane family of antibiotics and mycotoxins. The cyclase from *Fusarium sporotrichioides*, a homodimer of 45 kDa subunits,[4d] has been cloned[5a] and overexpressed in *Escherichia coli*.[5b] Experiments with stereospecifically labeled substrates have supported a cyclization mechanism in which FPP undergoes initial ionization and rearrangement to (3*R*)-nerolidyl diphosphate [(3*R*)-NPP][4a,6] (Scheme 1, path a). Rotation about the 2,3-bond followed by reionization of NPP (**3**) is thought to generate a cisoid allylic cation−pyrophosphate anion pair, which is then captured from the back face by the neighboring 6,7-double bond, resulting in generation of a bisabolyl cation **4**. Further cyclization of **4** followed by a 1,4-hydride shift gives **8**, from which a consecutive pair of 1,2-methyl migrations and deprotonation result in the formation of trichodiene. Although (3*R*)-NPP has been shown to be a viable substrate for trichodiene synthase[6,7] and experiments with both substrate[7,8ab] and intermediate[8c] analogs have provided support for this mechanistic scheme, none of the proposed enzyme-bound intermediates have ever been directly observed in the course of the cyclization.

Scheme 1



Recently, we described the results of experiments in which site-directed mutagenesis was used to alter amino acid residues 302−306 (DRRYR) of trichodiene synthase,[9] an arginine-rich domain suspected to play a role in binding of the pyrophosphate moiety of FPP.[10] Indeed, the R304K, Y305F, and Y305T mutants all showed substantial alterations in k_{cat} and/or K_m. Surprisingly, each of these mutants was also found by GC−MS analysis to convert FPP not only to trichodiene but to a mixture of varying quantities of 1−3 additional sesquiterpene hydrocarbons. We now report the identification of the anomalous products generated by two of these mutants and the results of isotopic labeling experiments that shed further light on both the natural and aberrant cyclization mechanisms.

Incubation of the Y305T mutant with FPP gave rise to a 4:4:4:1 mixture of trichodiene (**2**) and three additional sesquiterpene hydrocarbons, **5**, **6**, and **7**, each *m*/*z* 204, as revealed by GC−MS analysis.[11] Chromatographic purification of the crude hexane extract of a preparative incubation yielded 1.3 mg of **2**, 1.6 mg of **5**, and 1.4 mg of a 4:1 mixture of **6** and **7**. The 100.6 MHz ^{13}C NMR spectrum of **5** revealed the presence of two trisubstituted double bonds (δ 142.1, 133.6, 120.0, 118.5 ppm), establishing that **5** contained two rings, while the 400 MHz ^{1}H NMR spectrum showed the presence of a conjugated diene, based on the observed coupling constant, $J = 5.6$ Hz, between the olefinic protons [(d_{12}-pentane) δ 5.66 (d, $J = 5.6$ Hz, 1 H), δ 5.61 (dq, $J = 5.6$, 1.5 Hz, 1H)], as well as three quaternary methyl groups (δ 1.02, 1.01, and 0.82, each s, 3 H).[12] Detailed analysis of the ^{1}H−^{1}H COSY, INEPT, ^{1}H−^{13}C HETCOSY, and ^{13}C−^{13}C COLOC spectra led to the assignment of **5** as cuprenene, completely consistent with the reported NMR data for this metabolite.[13] Further chromatographic purification (SiO_2, pentane) of the mixture of **6** and **7** gave **6** which was found to contain one ring and three double bonds, based on the

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(11) In a typical preparative scale incubation, FPP, prepared as previously described,[6] (142 mg, 0.18 mM) was incubated with the Y305T mutant of recombinant *F. sporotrichioides* trichodiene synthase[9] (11.5 μM) from *E. coli* BL21(DE3)/pTS98-1(Y305T) in 1.6 L of Tris buffer (10 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol) for 16 h at 30 °C, before extraction with pentane and analysis and chromatographic purification of the sesquiterpene products.

(12) Although the two olefinic signals for **5** were unresolved in $CDCl_3$, recording of the ^{1}H NMR spectrum in d_{12}-pentane revealed the observed couplings.

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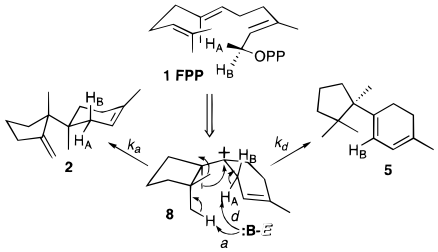
Scheme 2



observation of six olefinic signals in its ^{13}C NMR spectrum [δ 139.1, 133.8, 131.2, 123.6 (2C), 121.0]. The ^1H NMR spectrum confirmed the presence of the three olefinic protons [δ 5.41 (bs, 1 H), 5.09 (m, 2 H)] as well as four allylic methyl groups [δ 1.69 (s, 3 H), 1.66 (s, 3 H), 1.62 (s, 6 H)]. Extensive 2D NMR analysis, analogous to that carried out on **5**, and comparison with authentic spectra[14a] firmly identified **6** as (*Z*)-α-bisabolene. The absolute configuration of **6** was assigned as 4*R*, based on the observed $[\alpha]_D = -7 \pm 2°$ (*c* 0.07%, ethanol) [lit.[14a] $[\alpha]_D = -12.4°$ (*c* 1%, ethanol)]. Analysis of the ^1H and ^{13}C NMR signals for the minor component in 80% pure **6**, containing 20% **7**, established the presence of two trisubstituted double bonds (δ 5.07 and 5.33, each 1 H) and an exomethylene double bond (δ 4.73, 2 H) in **7**, implying that the minor component was also a monocyclic sesquiterpene. Detailed comparison with reported spectra[14b] confirmed the assignment of **7** as β-bisabolene. The absolute configurations of **5** and **7** were not assigned directly, but inferred from the known stereochemistry of trichodiene and that assigned to (−)-(*Z*)-α-bisabolene. Finally, GC−MS and NMR established that the Y305F mutant converted FPP to a 3:1 mixture of trichodiene (**2**) and cuprenene (**5**).

The formation of **5**, **6**, and **7** can be explained in each case by premature deprotonation of the normal carbocationic intermediates involved in the conversion of FPP to trichodiene (Scheme 1). Thus removal of a proton from either C-8 or C-14 of the bisabolyl cation **4** can produce (−)-(*Z*)-α-bisabolene (**6**) (path b) and β-bisabolene (**7**) (path c), respectively, in competition with normally expected electrophilic attack on the 10,11-double bond (path a). In a similar manner, cuprenene (**5**) can be generated by deprotonation of the bicyclic cation **8** (path d) in competition with the normal rearrangement-deprotonation of **8** to trichodiene (path a). Intriguingly, all three premature deprotonations might be mediated by the same enzymic base that is responsible for the final deprotonation step of trichodiene biosynthesis.

To further test this model, we determined the stereochemistry of the deprotonation that yields cuprenene (Scheme 2). Thus incubation of (1*R*)-[1-^{2}H]FPP[15] (**1**, $H_A = H$, $H_B = D$) with the Y305F mutant of trichodiene synthase gave trichodiene and cuprenene that each retained deuterium in the cyclohexenyl and the cyclohexadienyl moiety, respectively, as established by GC−EI/MS.[16] By contrast, parallel incubation of (1*S*)-[1-^{2}H]FPP[15] (**1**, $H_A = D$, $H_B = H$) gave deuterated trichodiene, as expected, but unlabeled cuprenene (Table 1). Control experiments were also carried out with unlabeled FPP (**1**, $H_A = H_B = H$) and

Table 1. GC−MS Analysis of Trichodiene and Cuprenene Derived from Deuterated FPP

	trichodiene (**2**)		cuprenene (**5**)	
	M^+	$[M^+ - C_7H_{11}]$	M^+	$[M^+ - C_6H_{13}]$
FPP	204	109	204	119
(1*R*)-[1-^{2}H]FPP	205	110	205	120
(1*S*)-[1-^{2}H]FPP	205	110	204	119
[1,1-$^2H_2$]FPP	206	111	205	120

with [1,1-$^2H_2$]FPP[15] (**1**, $H_A = H_B = D$) as substrates. The loss of the original 1-H_{si} proton of FPP in the formation of cuprenene is consistent with Schemes 1 and 2, based on the previously established retention of configuration at C-1 of FPP in the formation of trichodiene.[4b] Interestingly, cyclization of (1*S*)-[1-^{2}H]FPP also resulted in a significant suppression in the proportion of cuprenene to trichodiene, as a consequence of isotopically sensitive branching from the common intermediate **8**.[17] From the change in the ratio of trichodiene to cuprenene (from 3:1 to >7:1), the intrinsic isotope effect on the deprotonation step (^Dk_d) that generates **5** could be calculated to be ≥2.3.[17,18]

Although the actual functions of Tyr305 and of the adjacent Arg304 have not yet been established, it is reasonable to postulate that these residues are involved, directly or indirectly, in the binding of the pyrophosphate moiety of FPP.[9,10] Replacement of these residues may result in a small but important alteration in the precise positioning and folding of the substrate within the active site. Ionization of the aberrantly bound substrate could then lead to generation of abortive cyclization products by premature deprotonation of the various cationic intermediates in competition with the normal cyclization pathway. Site-directed mutagenesis is now one of the most powerful and widely used tools for the systematic investigation of protein structure and function. In the vast majority of cases, alteration of amino acid residues results in a simple degradation in the normal kinetic parameters, corresponding to an increase in K_m or decrease in k_{cat}, or both. In several instances, it has been possible to change the *substrate* specificity of an enzyme, as for example in the systematic mutation of trypsin to a give a hybrid protease with chymotrypsin-like activity.[19] The results reported here represent a novel example of the use of site-directed mutagenesis to alter the *products* of an enzyme reaction. Such experiments provide a powerful means to investigate fundamental issues of molecular recognition and catalytic control in terpenoid cyclizations and offer the possibility of eventually generating novel sesquiterpenes by rational engineering of known cyclases.

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(16) Crude cell lysate from *E. coli* BL21(DE3)/pTS100-10(Y305F)[9] (100 μL) was separately incubated with 162 μM FPP, 182 μM (1*R*)-[1-^{2}H]FPP, 192 μM (1*S*)-[1-^{2}H]FPP, and 218 μM [1-$^2H_2$]FPP in 1.0 mL of Tris buffer[9,11] for 3 h at 30 °C. The hydrocarbon products were extracted with HPLC grade pentane and purified on silica gel columns (3 cm) packed in Pasteur pipets overlaid with sodium sulfate (0.5 cm). The extracts of each incubation were concentrated *in vacuo* at 0 °C, and the concentrates (5 μL) were analyzed by GC−EI/MS.

(17) Isotopically sensitive branching has previously been used to perturb the proportion of products generated by the (−)-pinene synthases of sage (*Salvia officinalis*: Croteau, R. B.; Wheeler, C. J.; Cane, D. E.; Ebert, R.; Ha, H. *Biochemistry* **1987**, *26*, 5383−5389) and grand fir (*Abies grandis*: Wagschal, K.; Savage, T. J.; Croteau, R. *Tetrahedron* **1991**, *47*, 5933−5940) which convert geranyl diphosphate to mixtures of several cyclic and acyclic monoterpenes including (−)-α-pinene and (−)-β-pinene. Using this method, an intrinsic isotope effect of 2.4 has been determined for the deprotonation of a methyl to form (−)-β-pinene, while loss of a methylene proton to generate (−)-α-pinene showed an intrinsic isotope effect of 3.0. Methyl−methylene eliminations with k_H/k_D of 2.3−5.9 have been determined for limonene synthases from various sources: Pyun, H. J.; Coates, R. M.; Wagschal, K. C.; McGeady, P.; Croteau, R. B. *J. Org. Chem.* **1993**, *58*, 3998−4009.

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