Enzymatic Formation of Isochamigrene, a Novel Sesquiterpene, by Alteration of the Aspartate-Rich Region of Trichodiene Synthase

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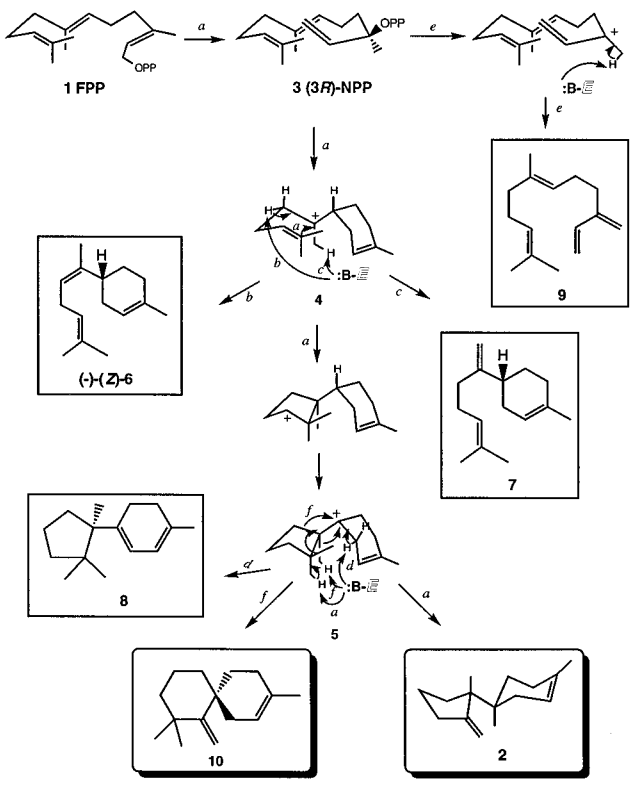
Received June 6, 1995

Sesquiterpene cyclases are among nature's most versatile and intriguing catalysts. Together these enzymes are responsible for the formation of more than 200 distinct sesquiterpenes carbon skeletons in plants and microorganisms. Each individual cyclase is capable of converting the universal acyclic precursor farnesyl diphosphate (FPP, **1**) to a distinct sesquiterpene.[1] Over the last 30 years, extensive studies have established a unified model for these complex transformations based on a common mechanism involving ionization of the allylic pyrophosphate ester followed by a precise sequence of intramolecular electrophilic addition reactions.[2] A major determinant which controls molecular diversity in product structure and stereochemistry is believed to be the precise folding of the FPP substrate at the cyclase active site. Although many of the mechanistic and stereochemical details of these mechanisms 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 carbon skeletal rearrangement, and ultimately quenches the positive charge.

Trichodiene synthase catalyzes the conversion of FPP to trichodiene (**2**), the parent hydrocarbon of the trichothecane family of antibiotics and mycotoxins.[4] The cyclase from *Fusarium sporotrichioides*, a homodimer of 45 kDa subunit, has been cloned[5a] and overexpressed in *Escherichia coli*.[5b] Experiments with labeled substrates,[4a,b,6] as well as with substrate[7] and intermediate[8] analogs, have provided support for the cyclization mechanism illustrated in Scheme 1 (pathway a)

Scheme 1. Formation of Multiple Sesquiterpenes by Cyclization of Farnesyl Diphosphate (**1**) by Mutant Trichodiene Synthases



in which FPP, folded in the manner shown, undergoes initial ionization and rearrangement to (3*R*)-nerolidyl diphosphate [(3*R*)-NPP, **3**]. Rotation around the 2,3-bond of **3** followed by reionization to generate a cisoid allylic cation-pyrophosphate anion pair allows cyclization to yield the bisabolyl cation **4**. Further cyclization of **4** followed by a 1,4-hydride shift gives **5**, from which a consecutive pair of 1,2-methyl migrations and a final deprotonation result in the formation of trichodiene (**2**). None of the proposed enzyme-bound intermediates have been directly observed with the wild-type enzyme, which produces trichodiene as the sole product.

Recent studies have been focused on modification of the enzyme active site by site-directed mutagenesis.[9] We have reported that various mutants of trichodiene synthase modified in an apparent active-site, arginine-rich domain produce mixtures of sesquiterpene hydrocarbons, including (−)-(*Z*)-α-bisabolene (**6**), β-bisabolene (**7**), and cuprenene (**8**), in addition to the natural cyclization product trichodiene (**2**).[10] Each of the aberrant cyclization products could in principle result from premature deprotonation of the normal cationic cyclization intermediates (Scheme 1, pathways b, c, and d). More recently we have extended these studies to mutants altered in a highly conserved aspartate-rich domain,[11] DDSKD, believed to mediate substrate binding, and possibly ionization, by chelation of the required divalent Mg^{2+} ion.[12] In fact, the D98E, D99E, and D102E mutants generated varying proportions of as many as five anomalous sesquiterpene hydrocarbons in addition to trichodiene (**2**), including **6**, **7**, and **8** as well as β-farnesene (**9**)

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S0002-7863(96)01897-5 CCC: $12.00

and a previously unidentified sesquiterpene **10**.[12] Replacement of Mg^{2+} by Mn^{2+} increased the proportion of aberrant products in each case, so that, for example, **10** consistuted more than 25% of the total product generated by the D98E mutant. We now report the isolation and identification of **10** as a novel sesquiterpene, which we have named isochamigrene.[13]

Chromatographic purification (SiO_2, pentane) of the crude pentane extract of a preparative-scale incubation of FPP, with trichodiene synthase D98E[14] gave two major fractions, I (R_f 0.84) shown to consist of a mixture of **6**, **7**, and **9,** and II (R_f 0.63) that included **2**, **8**, and **10**. Argentation chromatographic purification of fraction II (SiO_2/$AgNO_3$, pentane/ethyl ether 97:3 v/v) gave 3.7 mg of **2** ($R_f = 0.3$), 0.8 mg of **8** ($R_f = 0.2$), and 0.8 mg of **10** ($R_f = 0.12$) (HRMS *m*/*z* 204.1877, calcd for $C_{15}H_{24}$ 204.1878). The 100.6 MHz ^{13}C NMR spectrum of **10** revealed the presence of one exocyclic and one trisubstituted double bond (δ 161.3, 133.2, 120.4, and 107.3 ppm), establishing that **10** contained two rings, while the INEPT spectrum established that there were three methyl, six methylene, and two quaternary sp^3 carbon atoms. The 500 MHz ^1H NMR spectrum showed the presence of two exomethylene protons (4.96 (s, 1H), 4.91 (s, 1H)) and one additional olefinic proton (δ 5.30 (bs, 1H)) as well as two quaternary methyl groups (δ 1.12 (3H, s), 1.14 (3H, s)) and an allylic methyl (1.63 (3H, s)).

The connectivity in **10** was readily assigned by analysis of the combined results of HMQC, HMBC,[15] and NOE NMR spectra. Thus the HMQC spectrum showed that the methyl protons at δ 1.12 are attached to the carbon at 31.8 ppm (C-14) and that the methyl protons at δ 1.14 are attached to the carbon at 31.2 ppm (C-13). The latter methyl also shows an NOE with the olefinic methylene proton at δ 4.96. The allylic methyl protons (δ 1.63) are attached to C-15 (23.2 ppm). In the HMBC spectrum, the δ 1.12 methyl protons (H-14) are coupled by ^2J to a carbon at 36.6 ppm (C-10) and by ^3J connectivity to a carbon at 161.3 ppm (C-11) as well as to C-13 and C-9 (41.2 ppm). In the HMQC spectrum, the latter carbon is attached to 2 H-9 protons at δ 1.38−1.39. The HMBC spectrum also established that the H-15 methyl protons (δ 1.63) are coupled by ^2J to C-3 (133.1 ppm) and by ^3J to C-2 (120.1 ppm) and C-4 (27.6 ppm). In the HMQC spectrum, C-4 is directly attached to two protons at δ 1.82 and 1.87.

Turning to the olefinic protons, the HMQC spectrum established that H-2 (δ 5.3) is attached to C-2 (120.1 ppm) and also correlated the two H-12 protons with C-12 (107.1 ppm). In the HMBC spectrum, both H-12 protons have ^2J coupling with C-11 (161.3 ppm) and ^3J coupling to two additional carbons at 37.6 ppm (C-6) and 36.6 ppm (C-10). Taken together, these correlations unambiguously establish the key structural feature of the exomethylene double bond sandwiched between the quaternary carbons of the geminal methyl group and the spiro ring junction. Additional COSY, HMQC, and HMBC correlations showed ^3J connectivity between one of the H-1 protons (δ 2.06−2.12) and C-3 (133.1 ppm), C-7 (37.3 ppm), and C-5 (33.5 ppm). The full chemical shift correlations and NOE results are summarized in Figure 1. The absolute configuration of isochamigrene (**10**) was not directly determined but has been provisionally assigned on biosynthetic grounds.

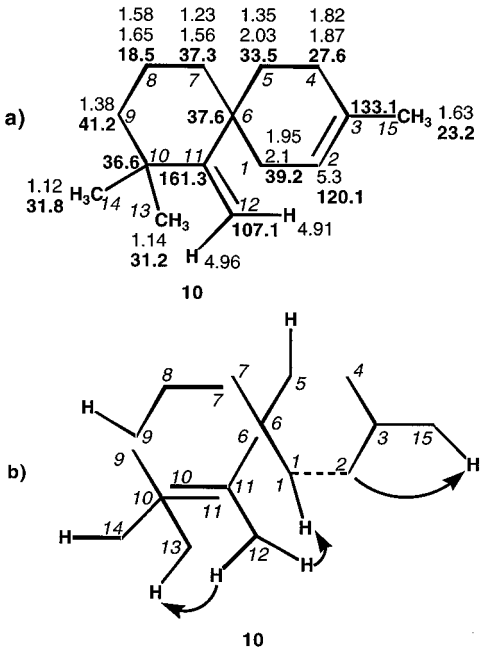

Figure 1. (a) ^1H (upper data, 499.84 MHz) and ^{13}C NMR (lower data, 125.7 MHz) NMR chemical shifts in ppm in $CDCl_3$. (b) Selected carbon connectivities observed from HMBC, HMQC (solid lines), ^1H−^1H COSY (dotted line), and NOEs (arrows) experiments.

Unlike the other aberrant cyclization products generated by the D98E[12] and other mutants[10] of trichodiene synthase, isochamigrene cannot be generated by simple deprotonation of one of the normal cationic cyclization intermediates. Instead, **10** can be formed by rearrangement of the late stage intermediate **5** through ring expansion followed by removal of a proton from C-12 (Scheme 1, pathway f). Nonetheless, the latter deprotonation might still be mediated by the same active site base which is believed to be responsible for formation of the remaining sesquiterpene hydrocarbon products.

The results reported here represent a novel example of the use of site-directed mutagenesis to produce an unnatural natural product.[16] Such discoveries raise the intriguing possibility that understanding the fundamental issues of molecular recognition and catalytic control in terpenoid cyclizations may eventually lead to rational engineering of known cyclases to generate additional novel sesquiterpenes.

Acknowledgment. This work was supported by a National Institutes of Health MERIT Award Grant, GM30301, to D.E.C. We would like to thank Dr. Meng Chen of Brown University for carrying out the GC/MS analyses and Dr. Francoise Sauriol of McGill University for assistance in acquiring the HMQC and HMBC spectra.

JA961897W

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(14) In a typical preparative scale incubation, FPP, prepared as previously described[8b] (109 mg, 0.11 mM), was incubated with the D98E mutant of recombinant *F. sporotrichioides* trichodiene synthase (15 μM) from *E. coli* BL21(DE3)/pBF01 in 2 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, followed by GC-MS and ^1H NMR analysis and chromatographic purification of the sesquiterpene products.

(15) HMBC spectra were recorded using two different mixing times of 70 and 100 ms.

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