

### Doubly Deuterium-Labeled Patchouli Alcohol from Cyclization of Singly Labeled [2-<sup>2</sup>H<sub>1</sub>]Farnesyl Diphosphate Catalyzed by Recombinant Patchoulol Synthase

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**Abstract:** Incubations of isotopically pure [2-<sup>2</sup>H<sub>1</sub>](*E,E*)-farnesyl diphosphate with recombinant patchoulol synthase (PTS) from *Pogostemon cablin* afforded a 65:35 mixture of monodeuterated and dideuterated patchoulols as well as numerous sesquiterpene hydrocarbons. Extensive NMR analyses (<sup>1</sup>H and <sup>13</sup>C NMR, <sup>1</sup>H homodecoupling NMR, HMQC, and <sup>2</sup>H NMR) of the labeled patchoulol mixture and comparisons of the spectra with those of unlabeled alcohol led to the conclusion that the deuterium label was located at positions (patchoulol numbering system) C5 (both isotopomers, ca. 100%) and C12 (minor isotopomer, 30–35%), that is, an approximately 2:1 mixture of [5-<sup>2</sup>H<sub>1</sub>]- and [5,12-<sup>2</sup>H<sub>2</sub>]-patchoulols. Low-resolution FIMS analyses and isotope ratio calculations further corroborated the composition of the mixture as mainly one singly deuterated and one doubly deuterated patchoulol. From a mechanistic point of view, the formation of [5,12-<sup>2</sup>H<sub>2</sub>]patchoulol is rationalized through the intermediacy of an unknown exocyclic [7,10:1,5]patchoul-4(12)-ene (**15-d<sub>1</sub>**), which could incorporate a deuterium at the C-12 position on the pathway to doubly labeled patchoulol. The corresponding depletion of deuterium content observed in the hydrocarbon coproducts,  $\beta$ -patchoulene and  $\alpha$ -guaiene (55% *d*<sub>0</sub>), identified the source of the excess label found in patchoulol-*d*<sub>2</sub>. Comparison of the PTS amino acid sequence with those of other sesquiterpene synthases, and examination of an active site model, suggested that re-orientation of leucine 410 side chain in PTS might facilitate the creation of a 2-pocket active site where the observed deuterium transfers could occur. The retention of deuterium at C5 in the labeled patchoulol and its absence at C4 rule out an alternative mechanism involving two consecutive 1,2-hydride shifts and appears to confirm the previously proposed occurrence of a 1,3-hydride shift across the 5-membered ring. A new, semisystematic nomenclature is presented for the purpose of distinguishing the three different skeletal structures of the patchoulane sesquiterpenes.

#### Introduction

The tricyclic tertiary alcohol (–)-patchoulol (patchouli alcohol, **2**) is a crystalline solid at room temperature first isolated by Gal in 1869.<sup>1</sup> This volatile sesquiterpene is the main component in patchouli oil (35–40%), an essential oil isolated from the East Indian shrub *Pogostemon cablin* (Blanco) Beth. (syn. *P. patchouli* Pellet). Patchouli alcohol contributes markedly to the characteristic odor of the oil<sup>2</sup> with a threshold of 0.93 ng/L air.<sup>3</sup> Patchouli oil is one of the most important natural fragrances utilized in the perfumery and cosmetic products industries.<sup>4</sup> In fact, well over 1200 tons of patchouli oil is produced annually by steam-distillation of the dried and

fermented leaves of *P. cablin*.<sup>5</sup> Since the first total synthesis of patchoulol by Büchi et al in 1962,<sup>6</sup> the rare tricyclo-[5.3.1.0<sup>5,10</sup>]undecane carbon skeleton of **2** and the biogenetically related sesquiterpene seychellene (**11**, see Figure 4, below) continue to be interesting synthetic challenges.<sup>7</sup>

Several mechanistic proposals have been suggested for the complex, enzyme-catalyzed cyclization of (*E,E*)-farnesyl diphosphate (FPP, **1**) to patchouli alcohol (**2**),<sup>8–10</sup> and experimental work on the biosynthesis of this novel bridgehead alcohol has been reported by Croteau et al.<sup>8</sup> and Akhila et al.<sup>9</sup> Native patchoulol synthase (PTS) from *P. cablin* (patchouli)<sup>11</sup> appears to carry out this multistep reaction through the intermediacy of five tertiary carbocations (**A–E**, Scheme 1) without detectable amounts of deprotonated intermediates.<sup>8,11</sup> On the basis of labeling results from incubation of [12,13-<sup>14</sup>C, 1-<sup>3</sup>H]FPP and

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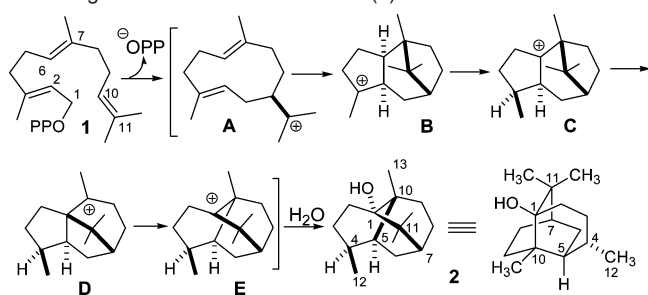
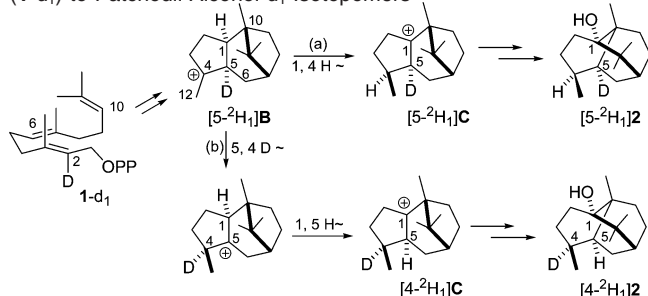
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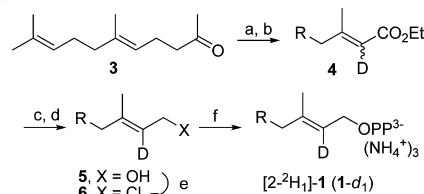
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**Scheme 1.** Mechanism Proposed for the Cyclization and Rearrangement of FPP to Patchoulol (**2**)<sup>8,11</sup>**Scheme 2.** Biosynthetic Pathways for Conversion of [2-<sup>2</sup>H<sub>1</sub>]FPP (1-*d*<sub>1</sub>) to Patchouli Alcohol-*d*<sub>1</sub> Isotopomers<sup>a</sup>

<sup>a</sup> Pathway (a) presents the accepted route to compound **2** via a 1,3-hydride shift (**B**→**C**).<sup>8,11</sup> Pathway (b) is a hypothetical variant of (a) involving two consecutive 1,2-hydride shifts to effect the **B**→**C** conversion.

[12,13-<sup>14</sup>C, 6-<sup>3</sup>H]FPP with cell-free extracts of patchouli leaves and degradation analyses of the resulting [<sup>14</sup>C, <sup>3</sup>H]patchoulol, Croteau and co-workers<sup>8</sup> concluded that the rearrangement step **B**→**C** takes place primarily by a 1,3-hydride shift (path a, Scheme 2) instead of an ostensibly less strained alternative involving two consecutive 1,2-hydride shifts (path b). Sequential 1,2-hydride shifts have been proposed to occur during the *in vivo* biosynthesis of  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -patchoulenes from (4*R*)-[4-<sup>3</sup>H, 2-<sup>14</sup>C]mevalonic acid in *P. cabli*.<sup>12</sup> However, the use of this particular labeled form of mevalonate should generate [2,6,10-<sup>3</sup>H, 4,8,14-<sup>14</sup>C]-FPP in the plant, and therefore does not distinguish between the different hydride shift alternatives.

Of five sesquiterpene synthase genes from *P. cablin* recently cloned and functionally expressed, one (PatTps177) was char-

**Scheme 3**<sup>a</sup>

<sup>a</sup> R = geranyl. Reagents, conditions, and yields: (a) LiC≡COEt, THF, -78 °C and (b) 10% D<sub>2</sub>SO<sub>4</sub>/D<sub>2</sub>O, -40 to 25 °C, 96% overall, 98.8% d-incorporation; (c) *i*-Bu<sub>2</sub>AlH, toluene, -78 °C, 97%; (d) isomer separation; (e) CH<sub>3</sub>SO<sub>2</sub>Cl, *s*-collidine, LiCl, DMF, 0 °C, 98%; (f) HOPP(NBu<sub>4</sub>)<sub>3</sub>, CH<sub>3</sub>CN, 25 °C; ion exchange, 58%.

acterized as patchoulol synthase (PTS).<sup>13</sup> The recombinant cyclase catalyzed the conversion of FPP to a mixture of patchoulol (37%) and a suite of sesquiterpene hydrocarbons generated in a composition closely resembling that of fresh leaf extracts. The availability of recombinant PTS and the capability of producing substantial amounts of patchoulol prompted us to undertake a reinvestigation of the mechanism by means of stable isotope labeling and NMR analysis. While 1,3-hydride shifts occur commonly in terpene synthase cyclization mechanisms,<sup>14</sup> we are unaware of any that take place across a 5-membered ring, ie requiring a transition state that would seem to resemble a highly strained bicyclo[2.1.1]pentane.

PTS-catalyzed cyclization of FPP substrate labeled with deuterium at C2 would afford patchoulol labeled at C5 if a 1,3-hydride shift occurred (pathway a in Scheme 2), or alternatively the label would move to C4 if two 1,2-hydride shifts had taken place (pathway b in Scheme 2). The location of deuterium in labeled patchoulol could be readily determined by high field NMR analyses. This paper provides evidence supporting the 1,3-hydride shift pathway (a), and the remarkable finding that some 35% of the labeled patchouli alcohol product derived from [2-<sup>2</sup>H<sub>1</sub>]FPP by the action of recombinant PTS is actually doubly labeled at C5 and C12.

## Results and Discussion

**Synthesis of [2-<sup>2</sup>H<sub>1</sub>]farnesyl Diphosphate.** The synthesis of [2-<sup>2</sup>H<sub>1</sub>]farnesol (**5**) and its corresponding diphosphate (**1-d**<sub>1</sub>) is outlined in Scheme 3. Addition of ethoxyacetylde to geranylacetone followed by hydrolysis with D<sub>2</sub>SO<sub>4</sub>/D<sub>2</sub>O<sup>15</sup> afforded a mixture of ethyl esters **4** (96%) that was reduced to a 1:1 mixture of *cis* and *trans* deuterated farnesols from which essentially pure (*E,E*)-[2-<sup>2</sup>H<sub>1</sub>]farnesol (**5**) was isolated by chromatography. The 500 MHz <sup>1</sup>H NMR spectrum showed the total absence (>98 atom % <sup>2</sup>H) of the diagnostic <sup>1</sup>H NMR triplet (*J* = 7.0 Hz) centered at 5.413 ppm and assigned to the vinyl hydrogen at C2 of unlabeled (*E,E*)-farnesol. The 77 MHz <sup>2</sup>H NMR spectrum of [2-<sup>2</sup>H<sub>1</sub>]farnesol (**5**) revealed a single olefinic resonance at  $\delta$  5.469 ppm (broad singlet) locating directly the deuterium label and proving the absence of detectable amounts of label at other positions. In addition, the doublet (*J* = 7.0 Hz) centered at  $\delta$  H

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**Table 1.**  $^{13}\text{C}$  (125 MHz) and  $^1\text{H}$  (600 MHz) NMR Chemical Shifts, Multiplicities,  $J$  Values, and Assignments for Patchoulol (**2**) in  $\text{CDCl}_3$ 

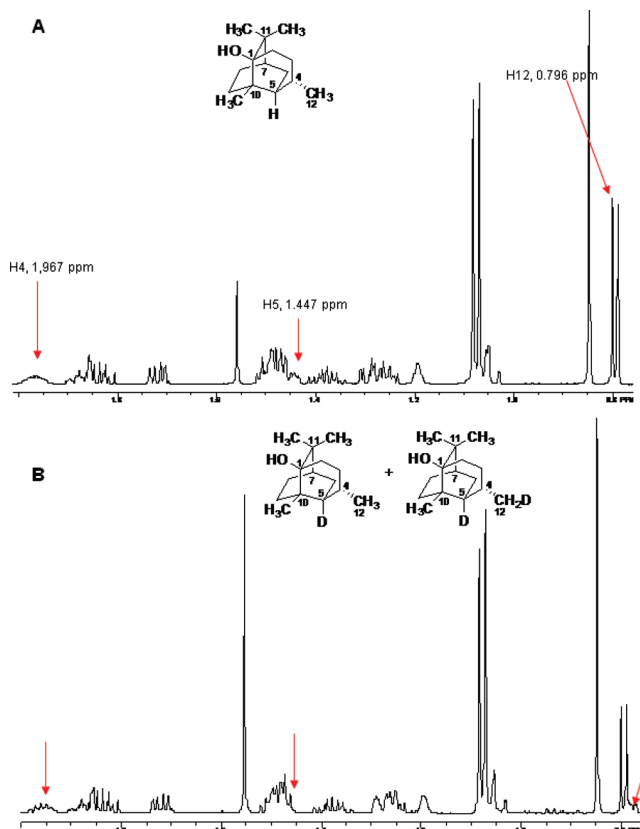
| assignment <sup>a</sup> | $\delta^{13}\text{C}^b$ | multiplicity <sup>a,c</sup> | $\delta^1\text{H}^{a,b}$                     |
|-------------------------|-------------------------|-----------------------------|----------------------------------------------|
| C1                      | 75.64                   | C                           |                                              |
| C2                      | 32.64                   | $\text{CH}_2$               | 1.511 dd (5.9, 3.6)<br>1.720 dd (12.6, 5.6)  |
| C3                      | 28.56                   | $\text{CH}_2$               | 1.373 dt (12.3, 5.9)<br>1.481 t (5.4)        |
| C4                      | 28.07                   | CH                          | 1.967 dq (6.6, 3.0)                          |
| C5                      | 43.66                   | CH                          | 1.447 ddd (8.7, 5.4, 3.0)                    |
| C6                      | 24.29                   | $\text{CH}_2$               | 1.298 dd (10.8, 3.6)<br>1.833 dd (11.4, 6.6) |
| C7                      | 39.04                   | CH                          | 1.195 br singlet                             |
| C8                      | 24.53                   | $\text{CH}_2$               | 1.268 dt (10.2, 3.0)<br>1.467 m              |
| C9                      | 28.81                   | $\text{CH}_2$               | 1.043 t (13.8)<br>1.821 dd (11.4, 7.2)       |
| C10                     | 37.63                   | C                           |                                              |
| C11                     | 40.08                   | C                           |                                              |
| C12                     | 18.52                   | $\text{CH}_3$               | 0.796 d (6.6)                                |
| C13                     | 20.62                   | $\text{CH}_3$               | 0.848 (s)                                    |
| C14                     | 26.81                   | $\text{CH}_3$               | 1.070 (s)                                    |
| C15                     | 24.27                   | $\text{CH}_3$               | 1.082 (s)                                    |

<sup>a</sup> Determined from HMQC 2D NMR experiments. For carbon numbering see Scheme 1. <sup>b</sup> In agreement with ref 18. <sup>c</sup> Determined from DEPT experiments.

4.15 ppm for the two C1 allylic protons was replaced by a sharp singlet (4.14 ppm) in the  $^1\text{H}$  NMR spectrum of  $[2\text{-}^2\text{H}_1]\text{farnesol}$ . Low resolution FI MS analyses of both unlabeled and labeled farnesols and isotopic ratio calculations confirmed a deuterium content of 98.8% in the labeled compound. Conversion of alcohol **5** to the corresponding allylic chloride **6** following Meyer's conditions,<sup>16</sup> and  $\text{S}_{\text{N}}2$  displacement with tris(tetrabutylammonium) pyrophosphate in  $\text{CH}_3\text{CN}$ <sup>17</sup> afforded diphosphate **1-d**<sub>1</sub> as the trisammonium salt in 58% overall yield after ion exchange and recrystallization from MeOH at 0 °C. The deuterated FPP was characterized by satisfactory  $^1\text{H}$  and  $^{31}\text{P}$  NMR spectra.

Complete assignments of the  $^1\text{H}$  (600 MHz) and  $^{13}\text{C}$  (125 MHz) NMR spectra of authentic unlabeled patchoulol (Table 1) were made by two-dimensional  $^1\text{H}$ – $^{13}\text{C}$  chemical shift correlations acquired in the inverse mode (HMQC) and were confirmed by comparisons with the existing literature data.<sup>18,19</sup> Of particular importance are the assignments of the resonances corresponding to the methine protons C4 and C5 as well as the C12 methyl group. The assignments in Table 1 are consistent with those previously reported by Barton et al.<sup>18b</sup>

**Enzymatic Cyclization of (*E,E*)-[2- $^2\text{H}_1$ ]farnesyl Diphosphate.** A preparative-scale incubation of  $[2\text{-}^2\text{H}_1]\text{farnesyl PP}$  (**1-d**<sub>1</sub>) with recombinant PTS performed according to Deguerry et al.<sup>13</sup> (pH 7.4; 5 mM  $\text{MgCl}_2$ , 30 °C) afforded deuterated patchoulols (**2**) in ca. 13% yield following purification by silica gel chromatography. Initial 600 MHz  $^1\text{H}$  NMR spectra of the deuterated



**Figure 1.**  $^1\text{H}$  NMR spectra (600 MHz,  $\text{CDCl}_3$ ) of (A) unlabeled and (B)  $[2\text{-}^2\text{H}_1]\text{patchoulol}$ . The three arrows mark regions where the spectra in (A) and (B) differ as a consequence of deuterium labeling.

patchoulol (Figures 1 and 2) thus obtained clearly showed: (1) the complete absence of the characteristic signal corresponding to the C5 methine proton (1.447 ppm) and (2) a reduced integration for the C12 methyl doublet (0.796 ppm, 3H,  $[5\text{-}^2\text{H}_1]\text{-2}$ , 65%).<sup>20a</sup> Careful integrations in the high field region relative to the C13 methyl singlet (0.848 ppm, 3H,  $[5\text{-}^2\text{H}_1]\text{-}$  and  $[5,12\text{-}^2\text{H}_2]\text{-2}$ , 100%)) showed, in addition to the C12 methyl doublet mentioned above (Figure 2A), a new resonance at 0.778 ppm (2H,  $[5,12\text{-}^2\text{H}_2]\text{-2}$ , 35%) with the multiplicity of a doublet of triplets ( $J = 6.6$  and 1.8 Hz) (Figure 2B) assigned to a  $\text{CH}_2\text{D}$  group. The small upfield displacement ( $\Delta\delta -0.02$  ppm) is typical for a geminal deuterium isotope shift.<sup>21</sup> In  $^1\text{H}$  homodecoupling NMR experiments (500 MHz), selective and complete saturation of the downfield signal at  $\delta_{\text{H}}$  1.967 (H4, Table 1) resulted, as expected, in the observation of the characteristic C12  $\text{CH}_3$  as a singlet (0.793 ppm, 1.94 H) accompanied by a new singlet (0.775 ppm, 1.06 H) shifted 0.018 ppm upfield due to the presence of a geminal deuterium in the  $\text{CH}_2\text{D}$  group (Figure 2C).<sup>21</sup> Similarly, analysis of the HMQC spectrum of  $[^2\text{H}]\text{patchoulol}$  (**2**; see Supporting Information) showed that the cross peak to C5 (43.66 ppm) was absent, and in addition to the characteristic H12/C12 crosspeak at 18.52 ppm, a small new crosspeak (18.05) shifted upfield 0.47 ppm by the  $^2\text{H}^{13}\text{C}$  isotope effect<sup>22</sup> was also noticeable. These deductions were confirmed by the 77 MHz  $^2\text{H}$  NMR spectrum (Figure 3) of the PTS-

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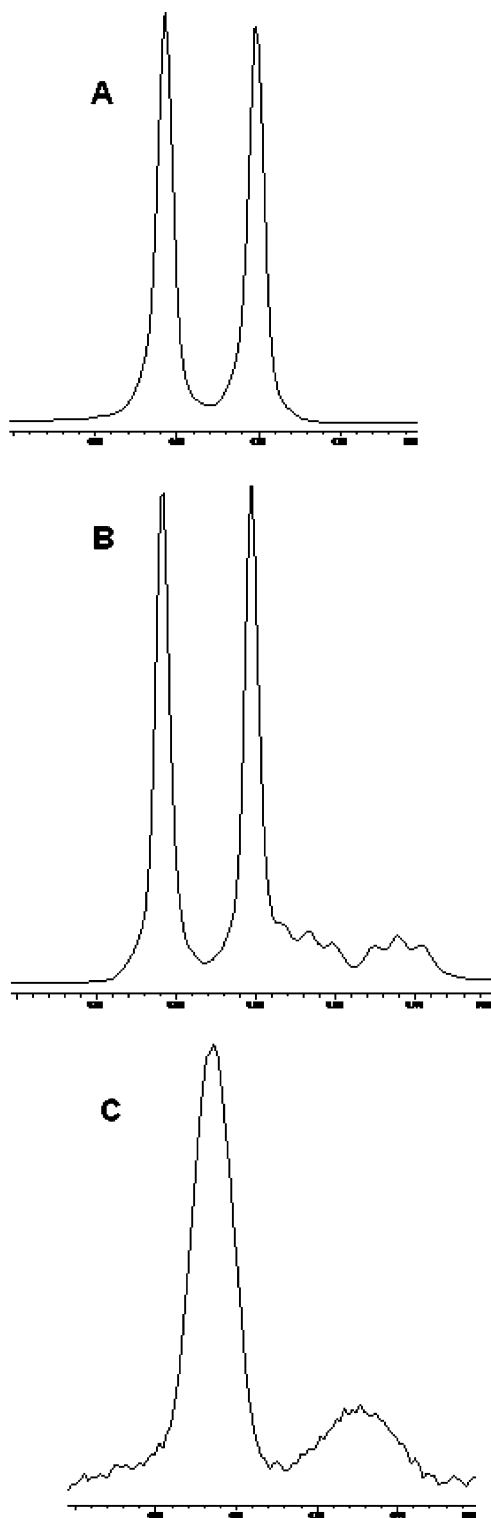
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(20) The % value in parenthesis refers to the relative populations of each  $[^2\text{H}]\text{patchoulol}$  calculated by integration of the individual (a)  $^1\text{H}$  NMR or (b)  $^2\text{H}$  NMR signals.

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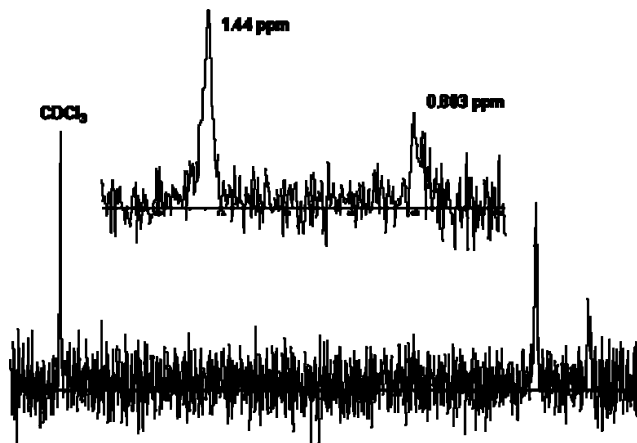
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**Figure 2.**  $^1\text{H}$  NMR spectra (600 MHz,  $\text{CDCl}_3$ ) of the H12 methyl doublet of unlabeled (A) and  $[\text{2-H}]$ -labeled patchouli (B), and 500 MHz  $^1\text{H}$  homodecoupled NMR spectrum (C) with selective and complete saturation of the downfield signal corresponding to H4 at  $\delta_{\text{H}}$  1.967 ppm.

generated  $[\text{2-H}]$ patchouli which displays two resonances at  $\delta$  1.44 (D at C5, 70%) and 0.80 (D at C12, 30%) ppm as broad singlets, respectively.<sup>20b</sup>

The  $^{13}\text{C}$  NMR spectrum (125 MHz,  $\text{CDCl}_3$ ) of the  $[\text{2-H}]$ patchouli product was very similar to that of unlabeled patchouli alcohol (Supporting Information). Noticeable, however, was the strong attenuation of the signal corresponding to C5 (43.66



**Figure 3.**  $^2\text{H}$  NMR spectrum (77 MHz,  $\text{CHCl}_3$ ) of the isomeric mixture of  $[\text{5-}^2\text{H}_1]$ - and  $[\text{5,12-}^2\text{H}_2]$ -patchoulols (**2**) generated by incubation of  $[\text{2-}^2\text{H}_1]$ FPP with recombinant PTS. (Inset) Expansion of the upfield region.

ppm). Instead, a new broad resonance was observed upfield ( $\Delta\delta$   $-0.54$  ppm)<sup>22</sup> at 43.12 ppm as a weak 1:1:1 triplet ( $J = 20$  Hz). Furthermore, in addition to the sharp peak at 18.52 ppm for the C12  $\text{CH}_3$  group, a new signal (18.05 ppm, weak 1:1:1 triplet ( $J = 21.2$  Hz)) shifted upfield by 0.47 ppm was observed and assigned to the  $\text{CH}_2\text{D}$  group.

The NMR evidence presented above strongly suggests that the incubation of isotopically pure  $[\text{2-}^2\text{H}_1]$ farnesyl diphosphate (**1- $d_1$** ) with recombinant PTS generated an inseparable mixture of two deuterated patchoulols ( $[\text{5-}^2\text{H}_1]$ - and  $[\text{5,12-}^2\text{H}_2]$ -**2**) in about 65:35 ratio. The total loss of the H5/C5 HMQC crosspeak at  $\delta$  1.447, F2 ( $^1\text{H}$ ) and  $\delta$  43.66, F1 ( $^{13}\text{C}$ ), and the apparent disappearance of the sharp C5  $^{13}\text{C}$  NMR signal at 43.66 ppm unambiguously locate the deuterium label at the C5 position in both  $[\text{2-H}]$ patchoulols, thus ruling out two sequential 1,2-H shifts (path (b), Scheme 2) as an alternative mechanism for the cationic transformation **B**  $\rightarrow$  **C**. As illustrated in Figure 2 (B and C) and Figure 3, the appearance of a second  $^1\text{H}$  NMR resonance for H12 centered at 0.778 ppm ( $^2\text{H}$  NMR  $\delta$  0.803 ppm, Figure 3) and the presence of a new  $^{13}\text{C}$  NMR signal at 18.05 ppm, both shifted upfield by 0.02 ppm and 0.47 ppm respectively,<sup>21,22</sup> locate a second deuterium label at the C12 position. Thus the minor PTS-generated  $[\text{2-H}]$ patchouli isotopomer (35%) corresponds to the doubly deuterated form  $[\text{5,12-}^2\text{H}_2]$ -**2**.

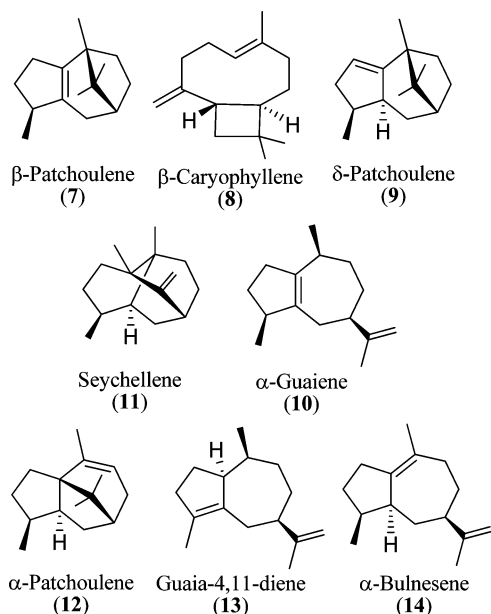
The partial incorporation (35%) of a second deuterium atom into the  $[\text{2-H}]$ patchouli product was also verified by careful analysis of the low resolution FI mass spectrum. The molecular ion region of labeled **2** displayed peaks at  $m/z$  222 (2%), 223 (100%), 224 (68%), 225 (34%), 226 (17%) and 227 (6%), and therefore indicated, as shown in Table 2, that the mixture is constituted mainly of  $d_1$  and  $d_2$  species in about 2:1 ratio (1.96:1). This result is in excellent agreement with the NMR integrations which show a composition mixture of  $d_1$  and  $d_2$  species in a 1.86:1 ratio (65:35).

**Analysis of the Hydrocarbon Fraction by  $^1\text{H}$  NMR Spectroscopy and GC-MS.** In addition to patchouli (**2**), recombinant PTS is known to produce at least 15 other sesquiterpenes.<sup>13</sup> In the present work, diagnostic  $^1\text{H}$  NMR signals<sup>23,24</sup> in combination with GC/MS data were used to identify 8 sesquiterpene hydrocarbons produced by recombinant PTS. Thus, preparative TLC on silica gel afforded two C15 hydrocarbon fractions according to GC-MS data (Table 2).  $^1\text{H}$  NMR (500 MHz) analysis of the less polar fraction (fraction

**Table 2.** Isotope Ratios of Sesquiterpene Hydrocarbons and Patchoulol Calculated from GC/MS (FI) Data for Products from Incubation of [2-<sup>2</sup>H]<sub>1</sub>FPP with Recombinant PTS

| compound and no.                              | rel. % <sup>a</sup> | GC t <sub>R</sub> <sup>b</sup> | d <sub>0</sub> | d <sub>1</sub> | d <sub>2</sub> | d <sub>3</sub> | d <sub>4</sub> |
|-----------------------------------------------|---------------------|--------------------------------|----------------|----------------|----------------|----------------|----------------|
| <b>Sesquiterpene hydrocarbons</b>             |                     |                                |                |                |                |                |                |
| β-Patchoulene (7)                             | 1.7                 | 17.8                           | 57.40          | 22.37          | 13.64          | 6.46           | 0.12           |
| Caryophyllene (8)                             | 3.5                 | 18.5                           | 4.80           | 84.96          | 4.96           | 5.28           | 0.00           |
| δ-Patchoulene (9) <sup>c</sup>                | 2.1                 | 19.2                           | 0.00           | 57.00          | 32.49          | 10.26          | 0.25           |
| α-Guaiene (10)                                | 29.4                | 19.8                           | 55.51          | 28.92          | 11.38          | 3.57           | 0.61           |
| Seychellene (11)                              | 25.2                | 20.0                           | 0.70           | 58.57          | 27.57          | 11.98          | 1.49           |
| α-Patchoulene (12)                            | 10.1                | 20.4                           | 0.78           | 57.50          | 29.50          | 11.85          | 0.61           |
| Unidentified                                  | 4.4                 | 21.2                           | 0.00           | 58.44          | 31.73          | 8.73           | 1.10           |
| Guaia-4,11-diene (13)                         | 10.4                | 21.7                           | 0.81           | 73.68          | 24.27          | 0.79           | 0.46           |
| α-Bulnesene (14)                              | 13.2                | 22.0                           | 0.63           | 56.80          | 28.01          | 10.29          | 4.28           |
| <b>Sesquiterpene alcohols</b>                 |                     |                                |                |                |                |                |                |
| [ <sup>2</sup> H]Patchoulol (2)               |                     |                                | 1.16           | 52.62          | 27.06          | 12.65          | 6.52           |
| Starting FPP (1-d <sub>1</sub> ) <sup>d</sup> |                     |                                | 1.14           | 98.86          |                |                |                |

<sup>a</sup> Estimated by measurements of GC and <sup>1</sup>H NMR peaks. (%) represent the relative percentage from the total of hydrocarbons. <sup>b</sup> GC conditions: see Experimental Part. <sup>c</sup> Tentative identification. <sup>d</sup> Deuterium content of farnesol-d<sub>1</sub>.

**Figure 4.** Structures of the PTS-generated sesquiterpenes identified in the hydrocarbon fractions by <sup>1</sup>H NMR spectroscopy in order of GC elution. The structural assignment of δ-patchoulene (9) is regarded as tentative.

1) showed the presence of three main constituents: α-guaiene (10, 33%),<sup>23a</sup> seychellene (11, 42%),<sup>23a</sup> and α-patchoulene (12, 21%)<sup>23a</sup> (Figure 4). Traces of other minor hydrocarbons accompanied the three major components, the more abundant of which showed four <sup>1</sup>H NMR resonances at δ 0.78 (s), 0.89 (s), 1.00 (s), and 0.98 (d, *J* = 6.5 Hz) ppm attributed to three tertiary and one secondary methyl groups, respectively. In addition, a broad singlet at δ 5.00 ppm assignable to an olefinic proton was observed. These <sup>1</sup>H NMR values at 500 MHz are in excellent agreement with the data previously reported for

synthetic δ-patchoulene at 60 MHz (0.78, 0.88, 0.95, 1.00, and 5.00 ppm).<sup>24</sup> Based on these considerations the structure of this product was tentatively identified as δ-patchoulene (9, ca. 4%), a minor sesquiterpene known to be present in patchouli oil,<sup>12,25</sup> but not previously recognized as a product of recombinant PTS.<sup>13</sup> Similarly, analysis of fraction 2 (more polar fraction) by <sup>1</sup>H NMR spectroscopy revealed, in addition to α-guaiene (26%) and seychellene (9%), and an unidentified sesquiterpene (9%) (Table 2), the presence of diagnostic resonances for (in order of GC elution): β-patchoulene (7, ca. 4%),<sup>23b</sup> β-caryophyllene (8, ca. 7%),<sup>23c</sup> guaia-4,11-diene (13, 18%),<sup>23d</sup> and α-bulnesene (14, 26%).<sup>23a</sup> The sesquiterpene profile observed in the present work is in excellent agreement with that previously reported by Deguerry et al.<sup>13</sup>

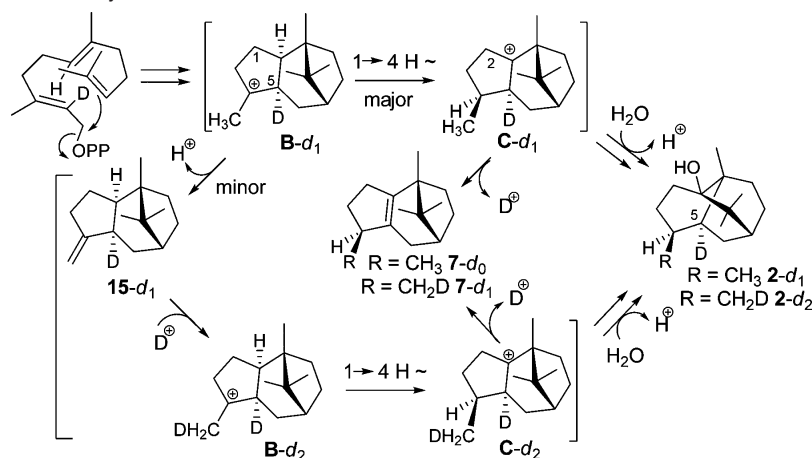
As shown in Table 2, incorporation of deuterium into the sesquiterpene hydrocarbon fractions took place at three distinctive and characteristic levels. While hydrocarbons 7 and 10 (Figure 4 and Table 2) were produced by the recombinant cyclase mainly as a 2:1 mixtures of d<sub>0</sub> and d<sub>1</sub> species, sesquiterpenes 9, 11, 12, 14, and an unidentified hydrocarbon as well as patchoulol 2 were generated mainly as 2:1 mixtures of d<sub>1</sub> and d<sub>2</sub> labeled forms. On the other hand, β-caryophyllene (8) and guaia-4,11-diene (13) were found to be essentially monodeuterated. It is clear that the enrichment of deuterium in labeled patchoulol is accounted for primarily by the depletion of deuterium in β-patchoulene (7, 57% d<sub>0</sub>) and α-guaiene (10, 56% d<sub>0</sub>). The enrichment of deuterium in seychellene (11, 59% d<sub>1</sub> + 28% d<sub>2</sub>) is very similar to that of patchoulol, consistent with their formation from a common bridgehead ion (Scheme 1, intermediate E).

It is worthwhile to comment here upon the confusing structural relationships and nomenclature for the patchoulane sesquiterpenes. As is evident from the structures of patchoulol (2) and the three patchoulene isomers in Figure 4, there are in fact three different skeletal structures for the “patchoulane” parent, independent of stereochemistry: deoxypatchoulol (tricyclo[5.3.1.0<sup>5,10</sup>]undecane), the dihydro derivative of β- and δ-patchoulene (7 and 9; tricyclo[6.2.1.0<sup>2,6</sup>]undecane), and dihydro α-patchoulene (12, tricyclo[5.3.1.0<sup>1,5</sup>]undecane).<sup>26</sup> Since all three tricycloundecane isomers have the same cyclodecane part structure (C1–C10) and they differ only in the locations of the one-carbon bridge (C11) and the zero carbon connections (0,<sup>5,10</sup> 0,<sup>2,6</sup> and 0<sup>1,5</sup>), we propose the following shorthand names to distinguish the three patchoulane carbon skeletons as follows [C11 bridge: ring fusion]: [1,7:5,10]-, [1,7:1,5]-, and [7,10:1,5]patchoulanes. Thus patchoulol (2), α-patchoulene (12), β-patchoulene (7), and δ-patchoulene (9) would be unambiguously named as [1,7:5,10]patchoul-1-ol, [1,7:1,5]patchoul-9(10)-ene, and [7,10:1,5]patchoul-1(5)-ene, and [7,10:1,5]patchoul-1(2)-ene, respectively.

The conversion of [2-<sup>2</sup>H]<sub>1</sub>(*E,E*)-FPP (1) to patchoulol (2) catalyzed by recombinant PTS proceeds with total retention of the deuterium label at C5 (patchoulol skeleton, or C2 farnesol skeleton). Thus pathway (b) in Scheme 2 is conclusively ruled out, and evidently intermediate carbocation B undergoes an apparently strained 1→3 hydride-shift to generate the cationic intermediate C en route to patchoulol (pathway a). This

- (23) α-Guaiene, seychellene, α-patchoulene and α-bulnesene, see: (a) Rakotonirainy, O.; Gaydou, E. M.; Faure, R.; Bombarda, I. *J. Essent. Oil Res.* **1997**, *9*, 321–327. β-Patchoulene, see: (b) Thomas, A. F.; Ozainne, M. *Helv. Chim. Acta* **1979**, *62*, 361–368. Caryophyllene, see: (c) Maurer, B.; Grieder, A. *Helv. Chim. Acta* **1977**, *60*, 2177–2190. Guaia-4,11-diene, see: (d) Saritas, Y.; Bulow, N.; Fricke, C.; König, W. A.; Muhle, H. *Phytochemistry* **1998**, *48*, 1019–1023.
- (24) δ-Patchoulene, see: Mookherjee, B. D.; Trenkle, R. W.; Ledig, W. O. *J. Agr. Food Chem.* **1974**, *22*, 771–773.

- (25) (a) Bunrathep, S.; Lockwood, G. B.; Songsak, T.; Ruangrunsi, N. *ScienceAsia* **2006**, *32*, 293–296. (b) Paknikar, S. K.; Veeravalli, J. *Indian J. Chem., Sect. B* **1980**, *19B*, 432. (c) Bure, C. M.; Sellier, N. M. *J. Essent. Oil Res.* **2004**, *16*, 17–19.
- (26) γ-Patchoulene ([1,7:1,5]patchoul-10(12)-ene) is the exocyclic 10(12) isomer of α-patchoulene (12).

**Scheme 4.** Proposed Reaction Pathway to the Deuterated Patchouls **2-*d*<sub>1</sub>** and **2-*d*<sub>2</sub>**

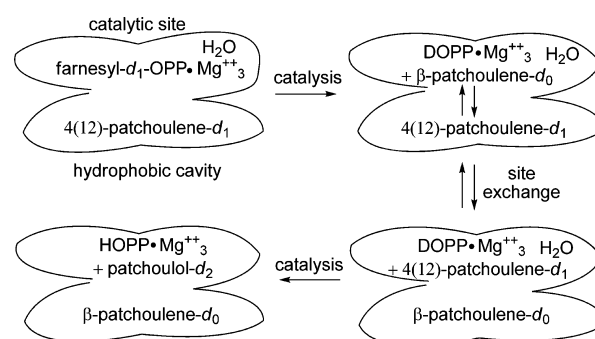
observation corroborates previous conclusions from biosynthetic studies by Croteau et al.<sup>8</sup> using the native enzyme, and is consistent with the mechanism presented in Scheme 1 as the biosynthetic pathway to patchouli alcohol (**2**). However, this scheme does not account for the formation of doubly deuterated patchoulol from singly labeled substrate.

**Mechanism for the Formation of Patchoulol-*d*<sub>2</sub>.** We propose that the second deuterium incorporated into the C12 methyl group of patchoulol occurs through a proton elimination followed by deuterium addition with the unknown sesquiterpene [7,10:1,5]patchoul-4(12)-ene-5-*d*<sub>1</sub> (**15-*d*<sub>1</sub>**)<sup>27</sup> as an intermediate (Scheme 4). Addition of D<sup>+</sup> to the 4(12) exocyclic double bond would regenerate intermediate **B-*d*<sub>2</sub>** in doubly deuterated form which could proceed through the remaining steps to [5, 12-<sup>2</sup>H<sub>2</sub>]patchoulol (**2-*d*<sub>2</sub>**). The low level of *d*<sub>3</sub> species present in [<sup>2</sup>H]patchoulol according to GC/MS analysis (Table 2, 12–13%) can be explained by a small amount of reversal of the **15-*d*<sub>1</sub>** → **B-*d*<sub>2</sub>** step by kinetically favored H<sup>+</sup> elimination giving some **15-*d*<sub>2</sub>** that could incorporate a second deuterium into the C12 methyl group. The elimination reactions forming the double bonds of  $\beta$ -patchoulene (**7**) and  $\alpha$ -guaiene (**10**) provide plausible sources of the deuterium in view of their isotope depletions. Since a direct, intermolecular deuterium transfer between intermediates **15-*d*<sub>1</sub>** and **C-*d*<sub>1</sub>** seems inconceivable, recombinant PTS presumably has the capacity to retain the D<sup>+</sup> label in the catalytic site long enough for re-entry of intermediate **15-*d*<sub>1</sub>**. Mechanistic schemes involving intramolecular transfers of protons have been proposed previously to rationalize observed migrations of deuterium label in the biosynthesis of taxadiene, abietadiene,

and fusicoccadiene catalyzed by the respective recombinant diterpene synthases.<sup>28</sup>

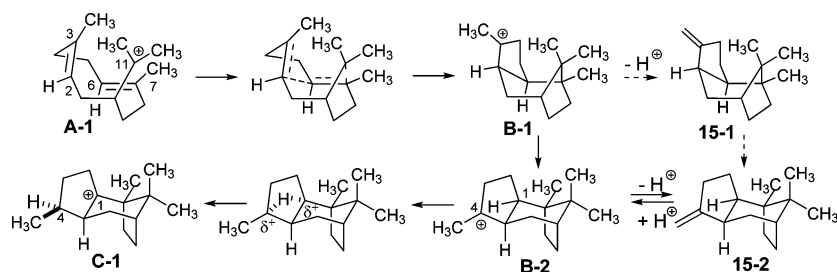
Köllner et al recently proposed that the multiple products generated by the maize sesquiterpene synthase TPS4 can be explained by the existence of two different active site pockets.<sup>29</sup> The alterations observed in product distributions from TPS4 mutant cyclases were consistent with this model in which monocyclic bisabolyl products are generated by cyclization of nerolidyl PP in Pocket I and bicyclic products are formed by proton-induced cyclization in Pocket II. We propose that the catalytic site of PTS similarly adjoins a hydrophobic pocket in which one (or more) of the cyclization products is (are) sequestered during catalysis. Thus, diffusion of a neutral intermediate and products may occur between the catalytic site and the proximal storage pocket.

Native PTS isolated from *P. cabin* by Munck and Croteau was determined by SDS-PAGE analysis to be a dimer composed of two apparently identical subunits of *M<sub>r</sub>* ≈ 40 000.<sup>11</sup> Thus another way to explain the double deuteration phenomenon would invoke channeling of proposed intermediate **15-*d*<sub>1</sub>** formed in one monomer active site to the other monomer active site where the D<sup>+</sup> donor was sequestered. This alternative scenario is similar to the two-pocket shown in Scheme 5, except that in this case the two active sites in dimeric PTS serve similar functions. The channeling of intermediates between two catalytic sites on the same enzyme has been demonstrated in the cases of the fungal cyclases, *ent*-kaurene synthase<sup>30</sup> and geosmin synthase.<sup>31</sup> However, this homodimer explanation seems to be excluded by the finding that recombinant PTS actually exists as a 64-kDa monomer (see Experimental Section), in agreement with previous results.<sup>13</sup>

**Scheme 5**

(27) Literature searches with SciFinder turned up reports of three other [7,10:1,5]patchoulene isomers besides  $\beta$ - and  $\delta$ -patchoulenes (**7** and **9**) and a related tertiary alcohol: [7,10:1,5]patchoul-3(4)-ene (patrinene),<sup>27a,b</sup> [7,10:1,5]patchoul-4(5)-ene,<sup>27c,d,e</sup> [7,10:1,5]patchoul-5(6)-ene,<sup>27d</sup> and the tricyclic alcohol [7,10:1,5]patchoul-5-ol,<sup>27d,f</sup> evidently an erroneous structure proposed for patchouli alcohol. However, the reliability of the structure assignments for these naturally occurring patchoulenes is open to question. No references to [7,10:1,5]patchoul-4(12)-ene (**15**) were found. However, [7,10:1,5]patchoul-1(5), 4(12)-diene was an intermediate in Büchi's total synthesis of patchoulol.<sup>6</sup> SciFinder references: (a) Peking Medical College, *Beijing Yixueyuan Xuebao* **1976**, *1*, 17–22; (b) Liu, M.; Chen, Y.; Bu, Y.; Fang, Y. *Shenyang Yaoxueyuan Xuebao* **1993**, *10*, 301–304; (c) Polovinka, M. P.; Korchagina, D. V.; Shcherbukhin, V. V.; Gatilov, Yu. v.; Rybalova, T. V.; Zefirov, N. S.; Barkhash, V. A. *Zh. Org. Khim.* **1995**, *31*, 214–219; (d) Treibs, W. *Liebigs Ann. Chem.* **1949**, *564*, 141–151; (e) Polovinka, M. P.; Korchagina, D. V.; Shcherbukhin, V. V.; Gatilov, Yu. v.; Rybalova, T. V.; Zefirov, N. S.; Barkhash, V. A. *Tetrahedron Lett.* **1995**, *36*, 8093–8096; (f) Esafov, V. I.; Novikov, N. I. *Zh. Obshch. Khim.* **1949**, *19*, 1344–1350.

Scheme 6



The pictorial model illustrated in Scheme 5 offers a credible explanation for the intermolecular deuterium transfer observed. Proposed intermediate **15-d<sub>1</sub>** formed in the catalytic site can be sequestered in the storage pocket while another [2-<sup>2</sup>H<sub>1</sub>]FPP substrate is bound in the catalytic site where it is converted to  $\beta$ -patchoulene (**7-d<sub>0</sub>**) or  $\alpha$ -guaiene (**10-d<sub>0</sub>**) by elimination of D<sup>+</sup>. Diffusive exchange of  $\beta$ -patchoulene (or  $\alpha$ -guaiene) with 4(12)-patchoulene **15-d<sub>1</sub>** would set the stage for generating the dideuterated products, provided that the exchange equilibrium of the deuterium with the external medium is slower than active site-storage pocket exchange and D<sup>+</sup> transfer to C12.

Inspection of the X-ray crystal structure of FPP synthase containing the inert substrate analogue dimethylallyl thiolopP and the normal cosubstrate isopentenyl PP in the catalytic site lead to the suggestion that the OPP·Mg<sub>3</sub><sup>2+</sup> leaving group accepts the 2R proton eliminated in the electrophilic coupling reaction.<sup>32</sup> Similarly it seems plausible that the OPP·Mg<sub>3</sub><sup>2+</sup> leaving group generated by the catalytic action of PTS could serve the same function. In a least-motion or minimal motion mechanism, the multidentate leaving group would be positioned close to C5 and C12 (corresponding to C2 and C15 of FPP). Hence the DOPP·Mg<sub>3</sub><sup>2+</sup> generated in the D<sup>+</sup> elimination forming  $\beta$ -patchoulene (or  $\alpha$ -guaiene) would be in a suitable position for deuterium transfer to C12 of 4(12)-patchoulene (**15-d<sub>1</sub>**), if the D/H exchange with the surrounding aqueous medium is relatively slow.

The obvious question arises whether the patchoulol product is actually formed by two different pathways operating in parallel as implied by Scheme 4. In other words, is [7,10:1,5]patchoul-4(12)-ene (**15**) an intermediate on a minor pathway to patchoulol, competing with the direct sequence **B** → **C** → **2** or is **15** in fact an obligatory intermediate? An alternative scenario is that all of the patchoulol is formed through the neutral 4(12)-patchoulene intermediate **15** and that the  $d_1 + d_2$  content of the patchoulol product formed from [2-<sup>2</sup>H<sub>1</sub>]FPP reflects a kinetic isotope effect on H/D exchange of DOPP·Mg<sub>3</sub><sup>2+</sup> with the surrounding aqueous medium. If a deuterium from DOPP·Mg<sub>3</sub><sup>2+</sup> can be incorporated into patchoulol by D<sup>+</sup> transfer to intermediate **15**, it seems highly likely that the analogous H<sup>+</sup> transfer would also be occurring. Thus, in this scenario the dominant pathway from FPP to patchoulol passes through 4(12)-patchoulene as an intermediate.

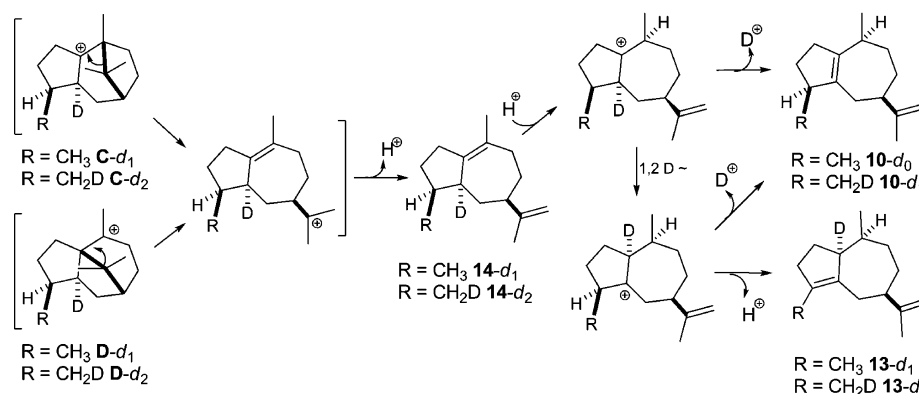
The two C–C bonds formed in the germacrenyl → patchoulenyl cyclization **A-1** → **B-1**, presumably proceed by a stepwise syn addition of the carbocation carbon (C11) and C2 across the 6,7 double bond (Scheme 6, FPP numbering). This exothermic process must involve considerable adjustment of bond angles as the two trans double bonds are converted to C–C single bonds in the rigid tricyclic ion **B-1**. A least-motion or minimal-motion process would form the central 6-membered ring initially in a boat conformation **B-1** with orbital alignment ill-suited for the C1 → C4 hydride transfer. Conformational inversion to a more stable chair form **B-2** would improve the orientation of the carbocation  $sp^2$  orbital for the hydride transfer, although further distortions of the 5-membered ring would seem necessary to attain the configuration of a bicyclo[3.1.1]hexane.

Since the proton removed from C12 to form the exocyclic double bond of **15** must be added again in a later step, the apparent necessity of passing through this neutral intermediate is puzzling. One possibility might be a proton elimination at C12 enforced by the nearby OPP·Mg<sub>3</sub><sup>2+</sup> counterion/base (not shown) in boat intermediate **B-1** to form the unstable conformation of **15-1** illustrated in Scheme 6. Perhaps the 5-membered ring of ion **B-2** is generated in a puckered conformation in the protonation of **15-2** so as to facilitate the 1→4 hydride transfer step. Alternatively the overall **B-1** → **C-1** transformation might take place by a mechanism other than the 1→4 hydride shift. This issue could be resolved by deuterium labeling at C1 (C6 of FPP). Also obscure are the reasons why pathway b via the more stereoelectronically favorable sequence of 1, 2-hydride shifts (5→4 and 1→5) does not occur.

The PTS-catalyzed formation of the bicyclic sesquiterpenes  $\alpha$ -guaiene (**10**), guaia-4,11-diene (**13**) and  $\alpha$ -bulnesene (**14**) (Figure 4) was previously proposed to occur by a proton-induced cyclization of the endocyclic double bonds of germacrene A, a minor hydrocarbon also detected in the product mixture.<sup>13</sup> However, with [2-<sup>2</sup>H<sub>1</sub>]FPP as the substrate, this route with protonation at C3 would generate **10-d<sub>0</sub>**, **13-d<sub>1</sub>**, and **14-d<sub>1</sub>** or by protonation at C10 would form **10-d<sub>1</sub>**, **13-d<sub>0</sub>**, and **14-d<sub>1</sub>**. In fact, GC/MS analyses in the present work established that the three guaienes are actually formed as mixtures of  $d_0 + d_1$  (2:1),  $d_1 + d_2$  (3:1) and  $d_1 + d_2$  (2:1) species, respectively, (see Table 2). This discrepancy in deuterium content can be explained by an alternative route to the bicyclic hydrocarbons independent of germacrene A. The deuterium label distribution present in these olefins can be explained by a later divergence from the pathway to patchoulol (**2**) after the bridged tricyclic ions **C** and **D**. As shown in Scheme 7, fragmentation of the C11–C10 or C11–C1 bridging bond of carbocation **C** or **D** followed by a PTS-mediated deprotonation at C12 would generate  $\alpha$ -bulnesene (**14**) as the required mixture of  $d_1 + d_2$  species. Reprotonation of the neutral olefin **14**, followed by a 1,2-deuteride shift would give rise to a new carbocation that could lose either a deuterium or a proton to generate  $\alpha$ -guaiene (**10**) or guaia-4,11-diene (**13**),

- (28) (a) Williams, D. C.; Carroll, B.; Jin, Q.; Rithner, C.; Lenger, S. R.; Floss, H. G.; Coates, R. M.; Williams, R. M.; Croteau, R. *Chem. Biol.* **2000**, *7*, 969–977. (b) Coates, R. M.; Ravn, M. M.; Jetter, R.; Croteau, R. *Chem. Commun.* **1998**, 21–22. (c) Toyomasu, T.; Tsukahara, M.; Kenmoku, H.; Anada, M.; Nitta, H.; Ohkanda, J.; Mitsuhashi, W.; Sassa, T.; Kato, N. *Org. Lett.* **2009**, *11*, 3044–3047.
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- (32) Hosfield, D. J.; Zhang, Y.; Dougan, D. R.; Broun, A.; Tari, L. W.; Swanson, R. V.; Finn, J. J. *J. Biol. Chem.* **2004**, *279*, 8526–8529.

**Scheme 7.** Proposed Biosynthetic Pathways to  $\alpha$ -Guaiene (**10**), Guai-4,11-diene (**13**), and  $\alpha$ -Bulnesene (**14**) via Bridge Fragmentation of Carbocations **C** and **D**



respectively (Scheme 7, lower right paths). Alternatively, the formation of olefin **10** could take place through a protonation/de deuteration sequence involving again a tightly bound  $\alpha$ -bulnesene intermediate (**14**) (Scheme 7, upper right path).

The amino acid sequences for patchoulol synthase (PTS; AAS86323) and the maize TPS4 (*Zea mays*) (TPS4; AY518310) enzyme were aligned with those for  $\gamma$ -humulene synthase (gHS; GenBank accession: AAC0528) and  $\delta$ -selinene synthase (dSS; AAC05727) from *Abies grandis*, and tobacco (*Nicotiana tabacum*) epiaristolochene synthase (EAS; AAA19216) using the publically available ClustalW (v1.4) multiple sequence alignment program in order to facilitate identification and discussion of conserved residues and domains (Figure 5). The PTS 3-dimensional structure was also modeled similarly to that described for the maize TPS4 enzyme by Köllner et al.,<sup>29</sup> except that the Modeler 6.2 software program<sup>35</sup> was used to thread the PTS amino acid sequence upon the structural coordinates for the *N. tabacum* EAS enzyme<sup>33</sup> (GenBank pdb file: 5EAT) and to energy minimize the final structural predictions. The EAS structure solved with the FPP analog farnesyl hydroxyphosphonate was used to position substrate docked into the various models. The PTS structural model was overlaid with that for the maize TPS4 enzyme<sup>29</sup> and the solved EAS structure<sup>33</sup> using the PYMOL software suite (www.pymol.org) (Figure 6).

In Figure 5, identical residues aligning in all 5 enzymes are back-highlighted in yellow, while residues of similar charge or polarity conserved among the enzymes are back-highlighted in green. Regions and residues of the enzymes suspected of playing particular roles in catalysis are outlined in colors as follows: The first DDXXD motif (I; outlined in red) positions a divalent cation near the entrance to the active site cavity and serves to fold the hydrocarbon portion of the FPP molecule into the active site pocket while anchoring the diphosphate substituent near the mouth of the pocket.<sup>33,36</sup> A second DDXXD motif (II; outlined in red), conserved only in  $\gamma$ -humulene and  $\delta$ -selinene synthases, has been suggested to provide a second FPP binding site and possible second active site facilitating the formation of large numbers of diverse reaction products emanating from these

enzymes.<sup>37</sup> The RXR motif (outlined in blue) is juxtaposed to the first DDXXD motif to assist in initiating ionization and sequestering of the diphosphate substituent outside the active site, and releasing the reactive carbocation intermediate into the active site pocket.<sup>14c</sup> The amino acid side chains of all the residues in the first DDXXD and RXR motifs are directly superimposed upon one another in the PTS and TPS4 structural models and the solved EAS structure.

The residues outlined in magenta have been shown by Köllner et al.<sup>29</sup> to be associated with the early steps in the reaction mechanism of the TPS4 enzyme, especially those steps involved in the initial ionization, isomerization and cyclization of the FPP substrate; the same steps correlated with activities putatively associated with pocket I of the TPS4 enzyme. The residues outlined in cyan have been associated with the second round of cyclization reactions occurring in the TPS4 enzyme,<sup>29</sup> and are suspected of either directly participating in the reaction mechanisms or possibly serving as a gatekeeper for intermediate movement between reaction pockets I and II. Although leucine 413 of TPS4 is conserved in sequence alignments with leucine 410 and 409 of PTS and EAS, respectively, its spatial orientation in the molecular model of PTS is directed away from the active site pocket in contrast to the inward and overlapping orientation found in the TPS4 and EAS structures (Figure 6). In contrast, threonine 301 of TPS4 occupies a similar spatial orientation as isoleucine 296 in EAS, but leucine 297 of PTS appears to extend a terminal methyl substituent across the active pocket sufficiently to distort the orientation of leucine 410 on the G2 helix. This may in turn distort the orientation of the entire G2 helix and the closely aligned J helix, two helices contributing directly to active site topology, and thus possibly providing for a larger reactive pocket or a second active site pocket.

## Conclusion

This work has established that recombinant patchouli alcohol synthase catalyzes the conversion of the singly labeled substrate [2-<sup>2</sup>H<sub>1</sub>]farnesyl diphosphate to doubly deuterated patchoulol ([5,12-<sup>2</sup>H<sub>2</sub>]-**2**) to the extent of ca. 35%, in addition to 65% of the singly labeled product bearing deuterium at C5 ([5-<sup>2</sup>H<sub>1</sub>]-**2**). The unexpected incorporation of label into the C12 methyl group implicates the unknown sesquiterpene [7,10:1,5]patchoul-4(12)-ene-*d*<sub>1</sub> (**15-d**<sub>1</sub>) as a neutral, enzyme-bound intermediate. The deuterium eliminated in producing the hydrocarbon coproducts,

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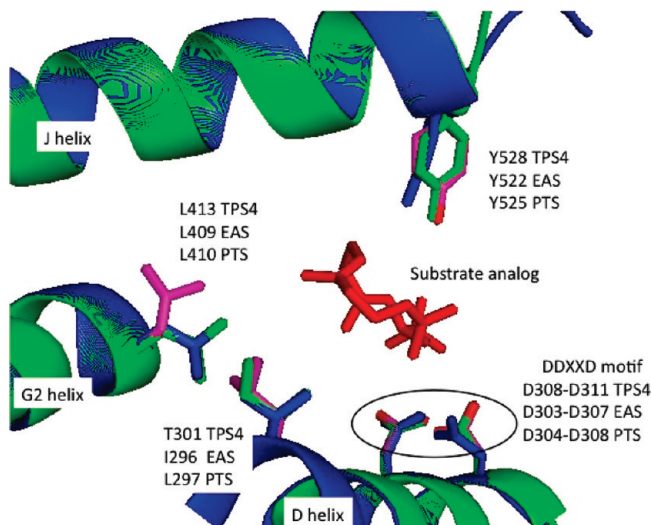
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|      |                                                     |     |
|------|-----------------------------------------------------|-----|
| gHS  | MAQISESVSPSTDLKSTESSITSNRHGNNWEDDRIQSLNSPYGAPAYQER  | 50  |
| dSS  | MAEISESSIPRR-----TGNHHGNVWDDLIHSLNSPYGAPAYEL        | 41  |
| TPS4 | MASPPAHRSSKAADDELP-KASSTFHPSLWGSFFLTQ--PPTAPQRANM   | 47  |
| EAS  | MASAAVANYEEEIVRP-----VADFSPSLWGDQFLSFSIDNQVAEKYIYA  | 45  |
| PTS  | MELYAQSVGVGAASRP-----LANFHPCVWGDKFIVYN--PQSCQAG-ER  | 42  |
| gHS  | SEKLIEEIKLLFLSDMDDSCNDSRDRLIKRLEIVDTVECLGIDRHFOPEI  | 100 |
| dSS  | LQKLIQEI KHLLEMEMD--DGDHDLIKRLQIVDTLECLGIDRHFEHEI   | 89  |
| TPS4 | KER-AEVLRRVRKVLKGS---TTDQLPETVNLILTQLRLGLGYYYENEI   | 93  |
| EAS  | QE--IEALKEQTRSMILLAT---GRKLADTLNLDIERLGISYHFEKEI    | 89  |
| PTS  | EE--AEELKVELKRELKEA---SDNYMRQLKMVDAIQRLGIDYLFVEDV   | 86  |
| gHS  | K-LALDYVYRCWNERGIGESRDSLKKDLNATALGFRALRLHRYNVSSGV   | 149 |
| dSS  | QTAALDYVYRWNEKGIGESRDSFSKDLNATALGFRALRLHRYNVSSGV    | 139 |
| TPS4 | D-KLLHQIYSNSDYN-----VKDLNLVSQRFYLLRKNQYDVPSPD       | 132 |
| EAS  | D-EILDQIYNQNSN-----CNDLCTSAIQFRLLRQHGFIISPEI        | 127 |
| PTS  | D-EALKNLFEMFADFCKN-----NHDMHATLSFRLLRQHGVRVSCV      | 128 |
| gHS  | LENFRDNGQFFCGSTVEEEGAEAYNKHVRCMLSLSRASNILFPGEKVME   | 199 |
| dSS  | LKNFKDENGKFFCNFTGEEG--RG-DKQVRSMLSLLRASEISFPGEKVME  | 186 |
| TPS4 | FLSKFTEEGGFACAAAD-----TRSLSLYNAAYLRKHGEEVLD         | 171 |
| EAS  | FSKFDENGKFKESLAS-----DVLGLNLNLYEASHVRTHADDILE       | 167 |
| PTS  | FEKFKD--G--KDGFKVPNE--DG---AVAVLE-FFEATHLRVHGEDVLD  | 168 |
| gHS  | EAKAFTTNYLKKVLAGR-EATHVDESLLGEVKYALEFPWHCSVQRWEARS  | 248 |
| dSS  | EAKAFTREYLNQVLAGHDVTDVQSLLEVKYALEFPWHCSVPWRWEARS    | 236 |
| TPS4 | EAISSTRLRLQDLLGRLLP---ESPFAKEVSSSLRTPFRVVGILEARN    | 217 |
| EAS  | DALAFSTIHLESAAPHL-----KSPLREQVTHALEQCLHKGVPRVETRF   | 211 |
| PTS  | NAFDFTRNYLESVYATL-----NDPTAKQVHNALNEFSFRRLPRVEARK   | 213 |
| gHS  | FIEIFGQIDSELKSNLSKKMLELAKLDFNQLQCTHQKELQIISRWFADS   | 297 |
| dSS  | FLEIYGHNSWLKSNINQKMLKLAKLDFNQLCKHHKEIQFITRWWR-DS    | 285 |
| TPS4 | YIPIYETEATRNEA-----VLELAKLNFNLQQLDFCEELKHCSAWWNEMI  | 262 |
| EAS  | FISSIYDKEQSKNN---VLLRFAKLDFNLLQMLHKQELAQVSRWWKDL    | 257 |
| PTS  | YISIYEQYASHHKG-----LLKLAKLDFNLVQALHRRLESDSRWWKTLO   | 258 |
| gHS  | SIASLNFYRKCYVEFYFWMAAAISEPEFSGSRVAFTKIAILMTMLDDLYD  | 347 |
| dSS  | GISQLNFYRKRHVEYYSWVVMCIFEPFSESRIAFAKTAILCTVLDLDDLYD | 335 |
| TPS4 | AKSKLTFVRDRIVEEYFWMNGACYDPPYSLSRILTKITGLITIIDDMFD   | 312 |
| EAS  | FVTTLPYARDRUVCEYFWALGVYFEPQYSQARVMLVKTISIMISIVDDTFD | 307 |
| PTS  | VPTKLSFVRDRIVESYFWASGSYFEPNYSVARMILAKGLAVLSLMDVVDYD | 308 |
| gHS  | THGILDQLKIFTEGVRRWDVSLVEGLPDFMKIAFEFWLKTSNELIAEAVK  | 397 |
| dSS  | THATLHEIKIMTEGVRRWDLSTDDLPDYIKIAQFFNTVNLIVEIVK      | 385 |
| TPS4 | THGTTEDCMKFAEAFGRWDESAIHLLPEYMKDFYILMLETQSFEDALGP   | 362 |
| EAS  | AYCTVKELEAYTDAIQRWIDINEIDRLPDYMKISYKAILDLKYDEKELSS  | 357 |
| PTS  | AYGTFEELQMFTDAIERWDASCLDKLPDYMKIVYKALLDVFEVDEELIK   | 358 |
| gHS  | AQGQDMAAYIRKNWERYLEAYLQDAEWIATGHVPTPDEYLNNGTNPNTGM  | 447 |
| dSS  | RQGRDMTTIV-KDCWKRYIESYLQEAEWIATGHIPTFNEYIKNGMASSGM  | 434 |
| TPS4 | --EKSYRVLYLKQAMERLVELYSKEIKWRDDDYVPTMSEHLQVSAETIAT  | 410 |
| EAS  | -AGRSHIVCHAIERMKEVVRNYNVESTWFIEGYMPPVSEYLSNALATTYVE | 406 |
| PTS  | -LGAPYRAYYGKEAMKYAARAYMEBAQWREQKHKPTTKYMKLATKTCGY   | 407 |
| gHS  | CVLNLIPLLLMGE-HLPIDILEQIFLPSRFHHLIELASRLNDDARDFQAE  | 496 |
| dSS  | CILNLIPLLLLDK-LLPDNILEQIHSPSKILDLELTGRIADDLKDFEDE   | 483 |
| TPS4 | IALTCSAYAGMGDMISIRKETFEWALSFPQFIRTFGSFVRLSNDVVSTKRE | 460 |
| EAS  | YYLATTSYLGMKS--ATEQDFEWLSKNPKILEASVIIICRVDDTATYEVE  | 454 |
| PTS  | ITLIILSCLGVEEGIVTKEAFDWFSRPPFIATLIITARLVNDITGHEFE   | 457 |
| gHS  | KDHGDLs-CIECYLKDHPSTVEDALNHVNGLLGNCLLEMNWKFLKKQDS   | 545 |
| dSS  | KERGEMASSLQCYMKENPESTVENALNHIKILNRSLEEFNWPFMK-QDS   | 532 |
| TPS4 | QTKDHSPTVHCYMKHEG-TMDDACEKIKELIEDSKWDMLEQSLA-LKG    | 508 |
| EAS  | KSRGQIATGIFCCMRDYGISTKE-AMAKFQNMATAWKDINEGLLR-PTP   | 502 |
| PTS  | KKREHVRTAVECYMEEHKVKQGE-VVSEFYNQMESAWKDINEGLLR-PVE  | 505 |
| gHS  | VPLSCKKYSFHVLSRSIQFMYNQ-GDGFSSISNKVIKQVQKVLIVPVPI   | 593 |
| dSS  | VPMCCKKFTFN-IGRGLQFIYKY-RDGLYISDKEVKDQIFKILVHQVPMEE | 581 |
| TPS4 | LPKVVPQLVFD-FSRTTDNMYRD-RDALTSS-EALKEMIQLLFVEPIPE   | 554 |
| EAS  | VSTFELTPILN-LARIVEVTYIHNLDGYTHPEKVLKPHIINLLVDSIKI   | 550 |
| PTS  | FPIPLLYLILN-SVRTLEVIYKE-GDSYTHVGPMQNIKQLYLHPVPY     | 552 |

**Figure 5.** Amino acid sequence alignment of patchoulol synthase (PTS) to two multireaction product sesquiterpene synthases,  $\gamma$ -humulene (gHS) and  $\delta$ -selinene synthases (dSS) from *A. grandis*, a maize synthase (TPS4) purported to have an active site divided into 2 pockets,<sup>29</sup> and epi-aristolochene synthase (EAS) from tobacco, with a single active site pocket as evident from crystallographic<sup>33</sup> and biochemical studies.<sup>34</sup>



**Figure 6.** Close-up view of putative active site residues influencing catalytic activity in patchoulol synthase (magenta highlighting), epi-aristolochene synthase (green highlighting) and TPS4 from maize (blue highlighting). This rendition attempts to accentuate the possible reorientation of leucine 410 in PTS such that it could facilitate creation of a second active site pocket.

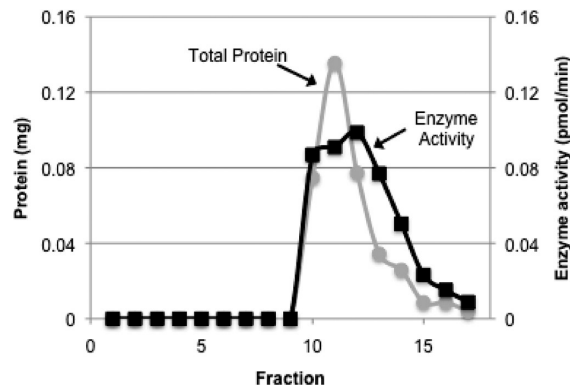
$\beta$ -patchoulene and  $\alpha$ -guaiane (**7** and **10**), predominantly in unlabeled form (55%  $d_0$ ) is considered the source of the excess label found in **2-d<sub>2</sub>**. A “two-pocket” catalytic site capable of sequestering **15-d<sub>1</sub>** and a  $D^+$  source (eg  $DOPP \cdot Mg_3^{2+}$ ) in close proximity is proposed to rationalize the double labeling. It seems reasonable to predict that [7,10:1,5]patchoul-4(12)-ene (**15**) is a naturally occurring sesquiterpene awaiting discovery.

The complete retention of deuterium at C5 in both labeled forms of patchoulol excludes a mechanism proceeding by two consecutive 1,2-hydride shifts (C5 to C4 and C1 to C5), and is consistent with the occurrence of the previously proposed 1,3-hydride shift (from C1 to C4)<sup>8</sup> through what would appear to be a stereoelectronically unfavorable transition state resembling a strained bicyclo[2.1.1]hexane.

## Experimental Section

**[2-<sup>2</sup>H<sub>1</sub>](2Z,6E)- and [2-<sup>2</sup>H<sub>1</sub>](2E,6E)-Farnesols (**5**).** A 1:1 mixture of cis,trans and trans,trans esters **4** (560 mg, 2.11 mmol) in dry toluene (5.0 mL) was reduced with *i*-Bu<sub>2</sub>AlH (1 M in hexane, 4.60 mL, 4.60 mmol) under N<sub>2</sub> for 3 h at  $-78^\circ\text{C}$ . Hydrolysis, extractions with 20% EtOAc/hexane, and purification by silica gel flash chromatography using 5% EtOAc/hexane as eluent gave pure trans,trans [2-<sup>2</sup>H<sub>1</sub>]farnesol **5** (168 mg, 34%): <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>)  $\delta$  5.10 (m, 2H), 4.14 (s, 2H), 1.95–2.14 (m, 8H), 1.68 (s, 6H), 1.59 (s, 6H), 1.25 (br s, 1H, OH); <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>)  $\delta$  139.7, 135.3, 131.3, 124.3, 123.7, 59.3, 39.7, 39.5, 26.7, 26.3, 25.7, 17.7, 16.2, 16.0. Noticeable was the apparent absence of the olefinic resonance of C2 (=CD-); <sup>2</sup>H NMR (77 MHz, CHCl<sub>3</sub>)  $\delta$  5.47 (broad s); MS (EI)  $m/z$  (rel. int.) 224 ( $M^+ + 1$ , 12), 223 ( $M^+$ , 62), 206 ( $M^+ + 1 - H_2O$ , 11), 205 ( $M^+ - H_2O$ , 53), 192 (100), 190 (44), 180 (51); HRMS (EI)  $m/z$  calcd for C<sub>15</sub>H<sub>25</sub>D<sub>1</sub>O ( $M^+$ ) 223.2046. Found: 223.2043.

**(2E,6E)-[2-<sup>2</sup>H<sub>1</sub>]Farnesyl Chloride (**6**).** Reaction of [2-<sup>2</sup>H<sub>1</sub>]farnesol **5** (168 mg, 0.75 mmol), with LiCl (500 mg, 11.8 mmol), *s*-collidine (500  $\mu$ L, 547 mg, 4.5 mmol), methanesulfonyl chloride (250  $\mu$ L, 170 mg, 1.5 mmol) in DMF (10.0 mL) for 3 h at  $0^\circ\text{C}$  and extractive workup gave 196 mg of a light-yellow oil. Purification by rapid flash chromatography on silica gel afforded 178.1 mg (98%) of pure deuterated chloride **6**: <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  5.09 (t,  $J = 6.8$  Hz, 2H), 4.10 (s, 2H), 1.93–2.16 (m, 8H), 1.73 (s, 3H), 1.68 (s, 3H), 1.60 (s, 6H); <sup>13</sup>C NMR (100 MHz,



**Figure 7.** Gel chromatography profile of recombinant PTS enzyme separated on a calibrated Sephadex G-75 column. A BSA molecular size standard of 66 kDa eluted in fraction 10 (not shown). PTS protein and enzyme activity eluted in fractions 10–12 with an apparent molecular size of 64–68 kDa, consistent with the monomeric size of 66 kDa calculated from the deduced amino acid sequence of the gene.

CDCl<sub>3</sub>)  $\delta$  142.7, 135.6, 131.4, 124.3, 123.4, 41.1, 39.6, 39.3, 26.6, 26.0, 25.7, 17.7, 16.1, 16.0. Noticeable is the absence of the olefinic resonance for C2 (=CD).

**(2E,6E)-[2-<sup>2</sup>H<sub>1</sub>]Farnesyl Diphosphate, Trisammonium Salt (**1-d<sub>1</sub>**).** Reaction of chloride **6** (178.1 mg, 0.74 mmol), and HOPP(NBu<sub>4</sub>)<sub>3</sub> (1.5 g, 1.7 mmol) in dry CH<sub>3</sub>CN (15 mL) in the presence of 4 Å molecular sieve powder (1.5 g) for 24 h at room temp according to a literature procedure,<sup>17</sup> dilution with CH<sub>3</sub>CN, filtration, and concentration under reduced pressure ( $40^\circ\text{C}$ ), cation exchange, and lyophilization gave the crude NH<sub>4</sub> salt as a white solid. Extractions with methanol followed by concentration, and recrystallization from MeOH at  $0^\circ\text{C}$  provided 187.1 mg (58%) of essentially pure diphosphate **1-d<sub>1</sub>**: <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD)  $\delta$  5.09 (broad t,  $J = 7.5$  Hz, 1H), 5.97 (app t,  $J = 6.0$  Hz, 1H), 4.41 (d,  $J = 5.0$  Hz, 2H), 2.02–1.84 (m, 8H), 1.73 (s, 3H), 1.58 (s, 3H), 1.55 (s, 3H), 1.49 (s, 3H), 1.48 (s, 3H); <sup>31</sup>P NMR (202 MHz, CD<sub>3</sub>OD)  $\delta$   $-8.78$  (broad d,  $J = 100.6$  Hz).

**Protein-Size Determination.** Recombinant PTS harboring a hexa-histidine amino terminal purification tag (see Supporting Information) was purified from 100 mL of *Escherichia coli* cells overexpressing the corresponding PTS gene as described previously (i.e., using Ni<sup>2+</sup> affinity column).<sup>13</sup> The isolated enzyme was concentrated to 100  $\mu$ L, dialyzed, and fractionated using a Sephadex G-75 column (0.9  $\times$  28.5 cm, 40–120  $\mu$ m particle size, Pfizer, New York, NY). The column was equilibrated (100 mM Tris, 50 mM NaCl, pH 8.0) and calibrated using the following standards (500–600  $\mu$ g): bovine serum albumin (BSA, 66.0 kDa), ovalbumin (43.0 kDa),  $\beta$ -lactoglobulin (35.0 kDa), or lysozyme (14.3 kDa). The molecular size of the recombinant PTS enzyme was then determined by chromatography on the calibrated Sephadex G-75 column (Figure 7) and shown to be ca. 66 kDa (monomer). Patchoulol synthase activity eluted from the column with a similar pattern, but slightly later than the bovine serum albumin (66.0 kDa) standard, which eluted in fraction 10. This observation is consistent with previous sizing results obtained from SDS-PAGE experiments (Figure 7).<sup>13</sup> The protein concentration of each fraction (0.43 mL) was determined with a protein assay reagent (Bio-Rad, Hercules, CA), and the PTS enzyme activity of each fraction measured in an enzyme assay with [1-<sup>3</sup>H] FPP as previously reported.<sup>13</sup> The enzyme showed a maximum specific activity of  $\sim 1$  pmol/min/mg enzyme, corresponding to the fractions 10–12.

**Incubation of (E,E)- [2-<sup>2</sup>H<sub>1</sub>]Farnesyl Diphosphate with Recombinant Patchouli Alcohol Synthase.** A typical large scale incubation experiment (500 mL) was set up as follows: [2-<sup>2</sup>H<sub>1</sub>]FPP (10 mg/mL) in 2.5 mL of buffer solution (250 mM Tris-HCl, pH 7.4; 50 mM MgCl<sub>2</sub>) was diluted with 50 mL of buffer solution, purified PTS synthase (ca. 1  $\mu$ g/ $\mu$ L, 20 mL), and distilled water

(427 mL). The reaction mixture was incubated in a 30 °C water bath overnight. Two such incubations were combined (1 L) and extracted with 15% EtOAc–hexane (2 × 1 L). The volume of the organic extracts was reduced to 20 mL by evaporation under reduced pressure. The crude extracts were shipped to the University of Illinois.

**Isolation of Sesquiterpenes.** The organic extract (20 mL) was evaporated using a stream of N<sub>2</sub>, and the residue (14.1 mg) was loaded into a preparative TLC (silica) plate. The plate was first developed with HPLC-grade pentane and then with 5% EtOAc–hexane to afford, in order of elution, a *m/z* 204 sesquiterpene hydrocarbon fraction (3.2 mg, 13.6%) and three *m/z* 222 sesquiterpene alcohol fractions (*Alcohol Fractions* 1 (3.2 mg, 12.5%), 2 (0.8 mg, 3%), and 3 (1.2 mg, 4.7%) according to GC-MS analysis. Initial <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) experiments revealed that *Alcohol Fractions* 1, 2, and 3 were composed of pure (>95%) patchoulols (**2**), [2-<sup>2</sup>H<sub>1</sub>]-nerolidol, and (*E,E*)-[2-<sup>2</sup>H<sub>1</sub>]-farnesol (**5**), respectively.

**Patchoulol (2, as 2:1 Mixture of [5-<sup>2</sup>H<sub>1</sub>]- and [5,12-<sup>2</sup>H<sub>2</sub>]-Isotopomers).** white solid; <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>) See Table 1 and Figure 1; <sup>1</sup>H NMR (500 MHz, C<sub>6</sub>D<sub>6</sub>) δ 1.97–1.90 (m, 1H), 1.88 (broad d, *J* = 12.5 Hz, 1H), 1.85 (m, 1H), 1.59 (dd, *J* = 12.5, 4.2 Hz, 1H), 1.40–1.11 (m, 7H), 1.10 (s, 3H), 1.07 (broad s, 1H), 1.00 (s, 3H), 0.97 (d, *J* = 13.5 Hz, 1H), 0.80 (s, 3H), 0.75 (dd, *J* = 6.5, 0.8 Hz, 3H, 12-CH<sub>3</sub>), 0.73 (d, *J* = 6.5 Hz, 2H, 12-CH<sub>2</sub>D); <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>) δ 75.62 (C1), 43.12 (1:1:1 t, *J* = 20 Hz, C5), 40.10 (C11), 39.08 (C7), 37.55 (C10), 32.66 (C2), 32.57 (C2, minor), 28.80 (C9), 28.57 (C3), 27.99 (C4), 27.91 (C4, minor), 26.83 (C14), 24.45 (C8), 24.30 (C6), 24.28 (C15), 20.60 (C13), 18.52 (C12), 18.05 (1:1:1 t, *J* = 21.2 Hz, C12 minor). <sup>2</sup>H NMR (77 MHz, CHCl<sub>3</sub>) δ 1.442 (broad s, D5), 0.803 (12-CH<sub>2</sub>D); LR(FI)MS *m/z* (rel. int.) 228 (1.4), 227 (6), 226 (17), 225 (34), 224 (68), 223 (100) 222 (2).

Further purification of the *m/z* 204 hydrocarbon fraction by preparative TLC on silica gel using hexane as developing solvent gave two fractions: *fraction 1* (ca. 1.5 mg) was composed of an approximate 10:8:5:1 mixture of sesquiterpenes **11**, **10**, **12** and **9**, respectively, according to <sup>1</sup>H NMR analysis (500 MHz, CDCl<sub>3</sub>). In the same manner, <sup>1</sup>H NMR analysis (500 MHz, CDCl<sub>3</sub>) of *fraction 2* (ca. 1.5 mg) revealed that this fraction was composed of (in addition to α-guaiene (**10**, 26%), seychellene (**11**, 9%) and an unidentified hydrocarbon (9%)) α-bulnesene (**14**, 26%), guaia-4,11-diene (**13**, 18%), caryophyllene (**8**, 7%) and β-patchoulene (**7**, ca. 4%).

**β-Patchoulene (7).** Partial <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) data from *fraction 2*: δ 0.93 (d, *J* = 6.7 Hz, 3H), 0.89 (s, 6H), 0.88 (s, 3H). LR(FI)MS *m/z* (rel. int.) 208 (2), 207 (15), 206 (30), 205 (55), 204 (100). The <sup>1</sup>H NMR data are in agreement with those reported for synthetic **7** at 360 MHz.<sup>23b</sup>

**β-Caryophyllene (8).** Partial <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) data from *fraction 2*: δ 5.31 (broad s, 1H), 4.94 (broad s, 1H), 4.82 (broad s, 1H), 1.61 (s, 3H), 0.99 (s, 3H), 0.97 (s, 3H). LR(FI)MS *m/z* (rel. int.) 208 (8), 207 (22), 206 (6), 225 (100), 204 (5.6). The <sup>1</sup>H NMR data are in agreement with those reported in ref 23c at 90 MHz.

**δ-Patchoulene (9).** Partial <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) data from *fraction 1*: δ 5.00 (broad s, 1H), 1.00 (s, 3H), 0.98 (d, *J* = 6.5 Hz, 3H), 0.89 (s, 3H), 0.78 (s, 3H). LR(FI)MS *m/z* (rel. int.) 208 (4), 207 (28), 206 (73), 205 (100).

**α-Guaiene (10).** Partial <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) data from *fraction 1*: δ 4.68 (broad s, 1H), 4.62 (broad s, 1H), 1.72 (s, 3H), 1.00 (d, *J* = 6.9 Hz, 3H), 0.93 (d, *J* = 6.8 Hz, 3H). LR(FI)MS *m/z* (rel. int.) 208 (2), 207 (10), 206 (30), 205 (67), 204 (100). The <sup>1</sup>H NMR data are in agreement with those reported in ref 23a at 200 MHz.

**Seychellene (11).** Partial <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) data from *fraction 1*: δ 4.79 (d, *J* = 1.5 Hz, 1H), 4.58 (d, *J* = 1.5 Hz, 1H), 0.96 (s, 3H), 0.83 (s, 3H), 0.74 (d, *J* = 6.9 Hz, 3H). LR(FI)MS *m/z* (rel. int.) 208 (6.5), 207 (29), 206 (64), 205 (100), 204 (1). <sup>1</sup>H NMR data are in agreement with those reported in ref 23a at 200 MHz.

**α-Patchoulene (12).** Partial <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) data from *fraction 1*: δ 5.07 (broad s, 1H), 1.62 (s, 3H), 0.95 (s, 3H), 0.90 (d, *J* = 6.9 Hz, 3H), 0.88 (s, 3H). LR(FI)MS *m/z* (rel. int.) 208 (4.7), 207 (27), 206 (63), 205 (100), 204 (1). The <sup>1</sup>H NMR data are in agreement with those reported in ref 23a at 200 MHz.

**Guaia-4,11-diene (13, Aciphyllene).** Partial <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) data from *fraction 2*: δ 4.67 (broad s, 1H), 4.59 (broad s, 1H), 1.75 (broad s, 3H), 1.57 (s, 3H), 0.75 (d, *J* = 7.1 Hz, 3H). LR(FI)MS *m/z* (rel. int.) 208 (1), 207 (8), 206 (49), 205 (100), 204 (1). The <sup>1</sup>H NMR data are in agreement with those reported in ref 23d at 400 MHz.

**α-Bulnesene (14).** Partial <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) data from *fraction 2*: δ 4.65 (s, 1H), 4.63 (s, 1H), 1.70 (s, 3H), 1.66 (s, 3H), 0.90 (d, *J* = 7.0 Hz, 3H). LR(FI)MS *m/z* (rel. int.) 208 (11), 207 (27), 206 (66), 205 (100), 204 (1). The <sup>1</sup>H NMR data are in agreement with those reported in ref 23a at 200 MHz.

**Unidentified.** LR (FI)MS *m/z* (rel. int.) 208 (5), 207 (25), 206 (71), 205 (100).

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**Supporting Information Available:** General experimental and instrumentation, detailed preparative procedures and characterization data, expression and purification of recombinant patchouli alcohol synthase, and reproductions of <sup>1</sup>H, <sup>2</sup>H, <sup>13</sup>C, and <sup>31</sup>P NMR spectra. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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