Dimethylallyl pyrophosphate is not the committed precursor of isopentenyl pyrophosphate during terpenoid biosynthesis from 1-deoxyxylulose in higher plants

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Contributed by Duiilio Arigoni, December 18, 1998

ABSTRACT Cell cultures of Catharanthus roseus were supplied with [2-13C,3-2H]deoxyxylulose or [2-13C,4-2H]1-deoxyxylulose. Lutein and chlorophylls were isolated from the cell mass, and hydrolysis of the chlorophyll mixtures afforded phytol. Isotope labeling patterns of phytol and lutein were determined by 1H NMR and 1H,2H-decoupled 13C NMR. From the data it must be concluded that the deuterium atom in position 3 of deoxyxylulose was incorporated into both isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate with a rate of 75% (with respect to the internal 13C label). The detected stereochemical signature implies that the label is located preferentially in the (E)-hydrogen atom of IPP. This preferential labeling, in turn, rules out dimethylallyl pyrophosphate as the compulsory precursor of IPP. In the experiment with [2-13C,4-2H]1-deoxyxylulose, the 13C label was efficiently transferred to the terpenoids whereas the 2H label was completely washed out, most probably after IPP formation as a consequence of the isomerization and elongation process. In addition, the data cast light on the stereochemical course of the dehydrogenation and cyclization steps involved in the biosynthesis of lutein.

The mevalonate pathway has been considered as the unique pathway of terpene biosynthesis for many years despite an abundance of experimental data that could not be explained (for reviews see refs. 1–4). Recently, independent studies done by the groups of Rohmer, Sahm, and D.A. showed that many bacteria generate isoprenoids by a different pathway (Fig. 1) from pyruvate (5–7). D.A.’s research group also could determine by 2H NMR and 1H,2H-decoupled 13C NMR. From the data it must be concluded that the deuterium atom in position 3 of deoxyxylulose was incorporated into both isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate with a rate of 75% (with respect to the internal 13C label). The detected stereochemical signature implies that the label is located preferentially in the (E)-hydrogen atom of IPP. This preferential labeling, in turn, rules out dimethylallyl pyrophosphate as the compulsory precursor of IPP. In the experiment with [2-13C,4-2H]1-deoxyxylulose, the 13C label was efficiently transferred to the terpenoids whereas the 2H label was completely washed out, most probably after IPP formation as a consequence of the isomerization and elongation process. In addition, the data cast light on the stereochemical course of the dehydrogenation and cyclization steps involved in the biosynthesis of lutein.

Enzymes catalyzing the formation of deoxyxylulose 5-phosphate from glyceraldehyde 3-phosphate and pyruvate have been characterized in Escherichia coli (13, 14) and Mentha piperita (15). More recently, an E. coli enzyme converting 1-deoxyxylulose 5-phosphate into 2-C-methylerythritol 4-phosphate has been reported (16). To date, no other intermediates on the way to isopentenyl pyrophosphate (IPP) (5) and dimethylallyl pyrophosphate (DMAPP) (6) have been identified.

Phytol (7, Fig. 2), β-carotene, and lutein (8) are biosynthesized in Catharanthus roseus from DMAPP and IPP stemming largely, if not exclusively, from deoxyxylulose (9). Specifically, DMAPP serves as a starter unit, which is converted into the common precursor geranylgeranyl pyrophosphate by sequential reactions with three IPP units. To obtain information on the nature and sequence of the unknown steps that lead from 1-deoxyxylulose to the two C5 building blocks of terpene biosynthesis we decided to follow the fate of individual hydrogen atoms of the precursor by using samples labeled both with 13C in position 2 and with 2H at either position 3 or position 4. This strategy makes it possible to follow the joint transfer of the two labels by the detection of the up-field shifts caused by the presence of deuterium on the 13C-signals of the enriched carbon centers.

EXPERIMENTAL PROCEDURES

Materials. [2-13C]pyruvate (99% 13C enrichment) was purchased from Isotec. [1-2H]glyceraldehyde was synthesized from 2,3-O-isopropylidene-d-glyceraldehyde acid methyl ester through reduction with LiAlD4 (98 atom % 2H) and subsequent Swern oxidation of the resulting alcohol. The isopropylidene group was removed by using Dowex 50/H+. [5-2H]D-glucose was prepared according to ref. 17. [2-13C]d-glyceraldehyde was prepared from [5-2H]D-glucose as described (18). [2-13C,3-2H]deoxyxylulose and [2-13C,4-2H]deoxyxylulose were prepared enzymatically by using [2-13C]pyruvate and [1-2H]d-glyceraldehyde, respectively, as substrates (19). The samples were purified by HPLC on a column of RPM Monosaccharide (8 μm, 300 × 7.8 mm, Phenomenex, Belmont, CA) using water as eluent and a refractometer as detecting system. The retention volume of deoxyxylulose was 12 ml. The following 1H NMR data were obtained for [2-13C,3-D]deoxyxylulose (ring-open form) in D2O: δ 2.04 (d, H-1, 13C coupling to C-2, 5.9 Hz), 3.42 (dd, H-5a, 13C coupling to H-5b, 11.2 Hz, 1H coupling to H-4, 7.1 Hz), 3.48 (dd, H-5b, 1H coupling to H-5a, 11.0 Hz, 1H coupling to H-4, 5.6 Hz), 3.95 (m, H-4). The following 13C NMR data were obtained for [2-13C,4-2H]deoxyxylulose in D2O: δ 2.04 (d, H-1, 13C coupling to C-2, 5.9 Hz), 3.41 (dd, H-5a, 1H coupling to H-5b, 11.2 Hz), 3.47 (d, H-5b, 1H coupling to H-5a, 11.2 Hz), 3.46 (d, H-3, 13C coupling to C-2, 3.4 Hz).

Plant Cell Culture and Isolation of Terpenoids. Cultures of C. roseus cells were grown as described (9). [2-13C,3-2H]1-deoxyxylulose (99% 2H enrichment, 93% 13C enrichment) or [2-13C,4-2H]1-deoxyxylulose (99% 13C enrichment, 75% 2H enrichment) have been identified. To whom reprint requests should be addressed.

Abbreviations: IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate.

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enrichment) was added to a final concentration of 44 mg/liter of culture fluid. The cultures were incubated for 7 days with continuous illumination. The cells were harvested (25 g, dry weight), and carotenoids and chlorophylls were isolated. Hydrolysis of chlorophylls afforded phytol as described (9). Further purification of phytol and lutein was achieved by HPLC on a column of Hypersil-RP18 (5 μm, 250 × 4.5 mm) using 95% (vol/vol) aqueous methanol as eluent. The effluent was monitored at 214 nm. The retention volumes were 10 ml for phytol and 6 ml for lutein. Typically, 0.4 mg of phytol and 0.06 mg of lutein were obtained per g of cell mass (dry weight), respectively.

**NMR Spectroscopy.** $^1$H and $^{13}$C NMR spectra of phytol and lutein were recorded at 17°C in CDCl$_3$ and $^2$H NMR spectra were recorded in CHCl$_3$ without lock by using a Bruker DRX500 spectrometer. $^2$H decoupling was achieved by using a lock switch unit. Two-dimensional experiments were performed according to standard Bruker software (XWINNMR 1.3). $^{13}$C Abundance in terpenoids was analyzed by quantitative NMR analysis as described (9).

**RESULTS**

Lutein and phytol were isolated from cell mass and analyzed by $^1$H, $^2$H, and $^{13}$C NMR spectroscopy. $^{13}$C NMR signal assignments for phytol were published earlier (9). $^1$H and $^{13}$C NMR signals of lutein and $^1$H NMR signals of phytol were assigned by two-dimensional correlated spectroscopy, nuclear Overhauser effect spectroscopy, heteronuclear multiple quantum correlation, and heteronuclear multiple bond correlation experiments (Tables 1 and 2).

In the experiment with [2-$^{13}$C,3-$^2$H]1-deoxyxylulose, four $^{13}$C signals of phytol (carbon atoms 3, 7, 11, and 15) and eight $^{13}$C signals of lutein (carbon atoms 1, 1’, 5, 5’, 9, 9’, 13, and 13’) displayed increased intensities, as expected on the basis of previous results (9). By comparison with the spectra of the unlabeled molecule, an average $^{13}$C content of 20.8 ± 1.8% was detected for the labeled centers (Tables 1 and 2).

As indicated in Figs. $3$ and $4$ each of the enriched signals is accompanied by high-field shifted satellites ($\delta$ = 26 to $-110$ ppm, Tables 1 and 2) shown to belong to the same molecule rather than to impurities by CH-correlated spectroscopy and correlated spectroscopy via long-range coupling (COLOC) experiments in which a $^1$H-$^{13}$C correlation of both the low- and high-field component with the same hydrogen atoms can be detected. The magnitude of the observed shifts is consistent with the presence of deuterium at positions adjacent to the $^{13}$C-enriched centers. The cotransfer of $^{13}$C and $^2$H can be assessed quantitatively from the relative intensities of the shifted vs. nonshifted signals after correction for the presence of molecules with natural $^{13}$C abundance.

The $^2$H NMR spectrum of phytol provides evidence for the presence of deuterium at C-4 ($\delta = 1.96$ ppm) and in one of the methyl groups ($\delta = 0.85$ ppm), which can be identified as C-16 because it is the only one in the molecule not derived from the methyl group of deoxyxylulose (9). The remaining signals at 1.24 and 1.04 ppm are the result of the pairwise overlap of signals belonging to two methylene groups adjacent to C-7 and C-8.

<table>
<thead>
<tr>
<th>Position</th>
<th>Chemical shift, $^a$ ppm</th>
<th>Preferred deoxysxylulose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^{13}$C</td>
<td>$^1$H</td>
</tr>
<tr>
<td>1</td>
<td>59.39</td>
<td>4.14 (d)</td>
</tr>
<tr>
<td>2</td>
<td>123.09</td>
<td>5.39 (t)</td>
</tr>
<tr>
<td>3</td>
<td>140.23</td>
<td>22.75</td>
</tr>
<tr>
<td>4</td>
<td>39.85</td>
<td>1.97 (m)</td>
</tr>
<tr>
<td>5</td>
<td>25.12</td>
<td>1.40 (m)</td>
</tr>
<tr>
<td>6</td>
<td>36.65</td>
<td>1.24 (m, $^2$H$_3$)</td>
</tr>
<tr>
<td>7</td>
<td>32.67</td>
<td>1.35 (m)</td>
</tr>
<tr>
<td>8</td>
<td>37.35</td>
<td>1.23 (m, $^2$H$_3$)</td>
</tr>
</tbody>
</table>

*Referenced to solvent signals at 7.24 and 77.0 ppm, respectively; multiplicities of the observed $^1$H NMR are indicated in parentheses (s, singlet; d, doublet; t, triplet; hp, heptet; m, multiplet)
†Calculated as the fraction of the $^2$H-shifted satellite signal in the global $^{13}$C NMR intensity of the adjacent atom. These values were used to calculate the cotransfer of $^{13}$C and $^2$H after correction for the presence of molecules with natural $^{13}$C abundance.
‡Assignments may be interchanged.

††Calculated as the fraction of the $^2$H-shifted satellite signal in the global $^{13}$C NMR intensity of the adjacent atom. These values were used to calculate the cotransfer of $^{13}$C and $^2$H after correction for the presence of molecules with natural $^{13}$C abundance.

†Assignments may be interchanged.
C-11, respectively (Fig. 5B). By comparison with the bioge- neically equivalent positions of lutein, the labeled methylene groups can be localized at C-8 and C-12. These methylene groups are known from the $^{13}$C NMR data to be labeled with the same amount of deuterium; the 3:1 ratio observed for the intensity of the signals at 1.04 ppm and 1.24 ppm in the $^2$H NMR spectrum must reflect the partitioning of the heavy atom among the two diastereotopic positions within each of the methylene groups. With reference to the spectroscopic work recently described by Eguchi et al. (20), the more intense high-field signal can be tentatively assigned to the Ha-positions (HSi-8; HSi-12) indicated in Fig. 6; this assignment is vindicated by the results obtained for the lutein probe generated in the same experiment.

Two overlapping signals at 1.0 ppm and 1.1 ppm in the $^2$H NMR spectrum of lutein (Fig. 7B) demonstrate the presence of deuterium in the 17$^9$ methyl group and in one of the two isochronous methyl groups linked to C-1. However, the $^{13}$C signals of the two groups are anisochronous and their stereochemical assignment already had been settled by Britton et al. (21). Hence, a choice in favor of the 17 methyl group can be made on the basis of the previous demonstration that this group (much as its 17$^9$ counterpart) stems specifically from C-3 rather than from the methyl group of the deoxyxylulose precursor (9). The $^2$H NMR spectrum of lutein also discloses the presence of deuterium at the 4 and 4$^9$ positions. The C-4 methylene group of lutein is biogenetically equivalent to C-12 of phytol and has the same deuterium content; the preponderance of the label in the Ha position (H-4b of lutein) is evident from the spectrum of the compound, but the low

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**Table 2. NMR analysis of lutein from a cell culture of C. roseus supplied with $[2-^{13}$C,3-2H]- and $[2-^{13}$C,4-2H]-deoxyxylulose**

<table>
<thead>
<tr>
<th>Position</th>
<th>$^{13}$C, ppm</th>
<th>$^2$H, ppm</th>
<th>Proffered deoxyxylulose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>37.09</td>
<td>20.01</td>
<td>$[2-^{13}$C,3-2H]</td>
</tr>
<tr>
<td>1'</td>
<td>34.01</td>
<td>19.28</td>
<td>$[2-^{13}$C,4-2H]</td>
</tr>
<tr>
<td>2</td>
<td>48.30</td>
<td>1.75(α,ddd)</td>
<td>$[2-^{13}$C,3-2H]</td>
</tr>
<tr>
<td>2'</td>
<td>44.60</td>
<td>1.34(α,dd)</td>
<td>$[2-^{13}$C,4-2H]</td>
</tr>
<tr>
<td>3</td>
<td>65.04</td>
<td>3.98(m)</td>
<td></td>
</tr>
<tr>
<td>3'</td>
<td>65.89</td>
<td>4.23(m)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>42.46</td>
<td>2.36(α,ddd)</td>
<td></td>
</tr>
<tr>
<td>4'</td>
<td>124.39</td>
<td>5.53(s)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>126.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5'</td>
<td>137.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>137.68</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6'</td>
<td>54.87</td>
<td>2.38(d)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>125.53</td>
<td>6.11(nd)</td>
<td></td>
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<tr>
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<td>125.71</td>
<td>5.41(dd)</td>
<td></td>
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<tr>
<td>8</td>
<td>138.47</td>
<td>6.11(nd)</td>
<td></td>
</tr>
<tr>
<td>8'</td>
<td>137.68</td>
<td>6.11(nd)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>135.68</td>
<td>21.83</td>
<td></td>
</tr>
<tr>
<td>9'</td>
<td>135.06</td>
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<td></td>
</tr>
<tr>
<td>10</td>
<td>130.77</td>
<td>6.11(nd)</td>
<td></td>
</tr>
<tr>
<td>10'</td>
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<td>6.11(nd)</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>124.77</td>
<td>6.11(nd)</td>
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</tr>
<tr>
<td>12,12'</td>
<td>137.53</td>
<td>6.34(d)</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>136.40</td>
<td>21.22</td>
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<tr>
<td>13'</td>
<td>136.48</td>
<td>21.46</td>
<td></td>
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<tr>
<td>14,14'</td>
<td>132.56</td>
<td>6.23(d)</td>
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<tr>
<td>14</td>
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<td></td>
</tr>
<tr>
<td>19'</td>
<td>13.11</td>
<td>1.89(s)</td>
<td></td>
</tr>
<tr>
<td>20,20'</td>
<td>12.81</td>
<td>1.95(s)</td>
<td></td>
</tr>
</tbody>
</table>

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$^*,$ $^†,$ $^‡$ See footnotes in Table 1.
intensity signal expected for the Hb position is hardly discernible in this spectrum, probably as a consequence of low resolution. The complex signal at 6–6.5 ppm is consistent with the presence of deuterium requested by the 13C NMR data for positions 8, 8', 12, and 12'. The labeling patterns of phytol and lutein from the experiment with [2-13C,3-2H]deoxyxylulose are summarized in Figs. 6 and 8.

In the experiment with [2-13C,4-2H]deoxyxylulose an average content of 16.1 ± 3.1% was observed for the 13C-enriched carbon atoms of phytol and lutein (Tables 1 and 2). The lack of high-field shifted satellite lines in the 13C NMR spectra (Figs. 3B and 4B) and the absence of signals in the 2H NMR spectra of phytol (Fig. 5C) and lutein demonstrate that the deuterium label of the precursor is not incorporated to any significant extent into the isoprenoids under investigation.

**DISCUSSION**

The labeling patterns observed for phytol (7) and lutein (8) in the experiment with [2-13C,3-2H]deoxyxylulose are consistent with the intermediate formation of a geranylgeranyl pyrophosphate (9), which had retained (with respect to the internal C-13 standard) 75% of the original deuterium label in its (E)-methyl group as well as in all other positions corresponding to the terminal methylene group of IPP (5); within the labeled methylene groups the label is distributed between the two heterotopic positions in a ratio of ca. 3:1 in favor of the Hα alternative (cf. Figs. 6, 8, and 9). This label distribution is preserved in phytol, whereas part of the deuterium is lost in later stages of lutein biosynthesis. The observed regiospecificity of deuterium incorporation matches the one recently reported for the formation of the octaprenyl side chain of ubiquinone in *E. coli* (22). In addition, the demonstration that deuterium label from C-3 of the precursor resides predominantly in the Hα-position of the labeled methylene groups implies a corresponding asymmetry for the label distribution within the terminal methylene group of the IPP intermediate. From the known stereochemical course of the chain elongation step in terpene biosynthesis (23) it is possible to reconstruct for the preponderant form of the intermediate the labeling pattern
indicated in Fig. 9. The observed partial scrambling of label is

in the form of 1-deoxyxylulose into 5-hydroxypentane-2,3-

is the most probable step in the well-documented

of Ha in one step and of Hb in the other, respectively (Fig. 8).

enzyme-catalyzed enolation of the precursor deserves specific

cells. Among the many remaining possibilities, the one of

carried out under identical conditions in the absence of plant

material was recovered unchanged at the end of an incubation

out by the result of a control experiment, in which the starting

Spontaneous enolization of the precursor in solution is ruled

the magnitude of the loss seems to exceed the figure, which can

sponsible for the scrambling of the deuterium label in IPP, but

sequence of the enzyme-catalyzed interconversion that is re-

and DMAPP, can be explained, in part, as a further conse-

quence of the enzyme-catalyzed enolization that is re-

ponsible for the scrambling of the deuterium label in IPP, but

the magnitude of the loss seems to exceed the figure, which can

be estimated from the observed degree of label randomization.

Spontaneous enolization of the precursor in solution is ruled

out by the result of a control experiment, in which the starting

material was recovered unchanged at the end of an incubation

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Among the many remaining possibilities, the one of

enzyme-catalyzed enolization of the precursor deserves specific

because it is known to operate in E. coli as the first and

most probably reversible step in the well-documented

transformation of 1-deoxyxylulose into 5-hydroxypentane-2,3-

Fig. 9. Biosynthetic origin of hydrogen atoms in geranylgeranyl

pyrophosphate (9) derived from IPP (5) and DMAPP (6) assembled

via the deoxyxylulose pathway. α and β indicate positions of deuterium

label. For details see Fig. 6 and text.

The sizable loss of deuterium (approximately 25%) from C-3

of the precursor, which accompanies the formation of both IPP

and DMAPP, can be explained, in part, as a further conse-

quence of the enzyme-catalyzed interconversion that is re-

ponsible for the scrambling of the deuterium label in IPP, but

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transformation of 1-deoxyxylulose into 5-hydroxypentane-2,3-

dione (24).

Because of the C2 symmetry of lycopene (11, Fig. 8), the last

acyclic precursor of lutein, each carbon center of lutein

corresponds biosynthetically to a pair of carbon centers of

the precursor, which are homotopically related and hence

distinguishable. As a consequence, any deuterium label appended

to these centers in the precursor will be distributed statistically

among the two corresponding centers of the derived lutein,

unless this label is affected specifically after the asymmetriza-

tion process. The identical or nearly identical deuterium

degrees detected in lutein for such positions is therefore not

fortuitous and should be viewed instead as a test for the

accuracy of the analytical tools that led to their detection. In

this perspective, it is clear that formation of both the A9

and the Δ12 double bonds of lycopene involves specific removal of

Hα atoms from phytoene (10), whereas the deuterium values

observed for C-8 and C-8′ in lutein are highly suggestive of a

doUBLE hydrogenation sequence involving specific removal of

Hα in one step and of Hβ in the other, respectively (Fig. 8).

These results match only in part previous findings on the

biosynthesis of carotenoids in Flavobacterium, which were

interpreted as evidence for an exclusive loss of Hα in each of

the four hydrogenation steps (25).

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accuracy of the analytical tools that led to their detection. In

this perspective, it is clear that formation of both the A9

and the Δ12 double bonds of lycopene involves specific removal of

Hα atoms from phytoene (10), whereas the deuterium values

observed for C-8 and C-8′ in lutein are highly suggestive of a

doUBLE hydrogenation sequence involving specific removal of

Hα in one step and of Hβ in the other, respectively (Fig. 8).

These results match only in part previous findings on the

biosynthesis of carotenoids in Flavobacterium, which were

interpreted as evidence for an exclusive loss of Hα in each of

the four hydrogenation steps (25).

In connection with the known origin of C-17′, which stems

from C-4 of IPP via the (E)-methyl group of DMAPP, it is now

possible to exploit the data pertaining to C-4′ of lutein for

reconstructing the stereochemic course of the cyclization

process responsible for the formation of the A′-ring of lutein.

Starting with the chair conformation indicated in Fig. 10,

addition of a proton triggers the formation of a cyclic cation,

in which Hα resides in an axial position meeting the stereo-

electronic requirement necessary for its subsequent elimina-

tion. Within the same scheme the alternative elimination of

Hβ, which is similarly oriented with respect to the adjacent

electronically deficient center, accounts for the formation of

an A-ring with the correct stereochanical arrangement of

the two biogenetically nonequivalent methyl groups at C-1.

In the experiment with [2,13C,4-2H]deoxyxylulose no deu-

terium transfer to phytol or lutein could be detected; the

absence of label that must be inferred in the Catharanthus

system for the DMAPP starter unit as well as for all the C3

units derived from IPP in the elongation process is in striking

contrast with the situation in E. coli, where it was shown that

during formation of the octaprenyl side chain of ubiquinone

deoxyxylulose from C-4 of deoxyxylulose is retained specifically

in the vinylic position of the DMAPP starter unit (23). This

finding suggests that (i) in both systems the deuterium from

C-4 of the precursor is incorporated stereospecifically into the

C-2 methylene group of IPP and subsequently lost in the

elimination step of the elongation process and (ii) the two

systems differ in the stereospecificity of their IPP-DMAPP

isomerase, the Catharanthus enzyme operating on the same

proton that is involved in the elongation step and the anom-

alous E. coli enzyme affecting instead the enantiotopic Hα

position (Fig. 9). Alternative explanations, e.g., independent

formation of IPP and DMAPP from a common intermediate

along the deoxyxylulose pathway, seem less plausible but

cannot be ruled out. It is therefore important to secure by

independent means that the C-4 hydrogen of the precursor is

indeed preserved during formation of IPP, because this re-
tention would impose a further stringent requirement on the

mechanism of the still unknown steps of the alternative

pathway.

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