Inhibition of the Non-Mevalonate l-Deoxy-D-xylulose-5-phosphate Pathway of Plant Isoprenoid Biosynthesis by Fosmidomycin

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Various bacterial and plastidic plant terpenoids are synthesized via the non-mevalonate 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway. The antibiotic and herbicidal compound fosmidomycin is known to inhibit growth of several bacteria and plants, but so far its mode of action was unknown. Here we present data which demonstrate that the DOXP pathway of isoprenoid biosynthesis is efficiently blocked by fosmidomycin. The results point to the DOXP reductoisomerase as the probable target enzyme of fosmidomycin.

Introduction

The alternative non-mevalonate 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway for isoprenoid formation is widespread in the plant kingdom (for review see Lichtenthaler \textit{et al.}, 1997a; Lichtenthaler, 1998). It is also present in various bacteria (Rosa Putra \textit{et al.}, 1998a). In higher plants, chloroplast-bound isoprenoids (carotenoids, phytol side-chain of chlorophylls, nonaprenyl side-chain of plastoquinone-9) are formed via the DOXP pathway (Lichtenthaler \textit{et al.}, 1997b; Schwender \textit{et al.}, 1997; Arigoni \textit{et al.}, 1997). Also the formation of lycopene in chromoplasts of ripening tomato fruits proceeds via this alternative DOXP pathway (Schwender and Lichtenthaler, 1998). The sterols of higher plants, in turn, are formed and accumulated in the cytosolic cell compartment via the classical mevalonate (MVA) pathway (Lichtenthaler \textit{et al.}, 1997a, b). The two cell compartments of plant isoprenoid biosynthesis – plastids and cytosol – possess two different IPP pathways: the plastidic DOXP pathway and the cytosolic MVA pathway (Lichtenthaler, 1998; Schwender \textit{et al.}, 1997). In addition to higher plants, we also found among eukaryotic algae (heterokontophyta, rhodophyta and chlorophyta) the formation of plastid-bound phytol and carotenoids according to the alternative DOXP pathway (Schwender \textit{et al.}, 1996; Disch \textit{et al.}, 1998). Isoprene, as the simplest plant isoprenoid and as a close derivative of IPP, is synthesized and emitted by many plants at high light conditions and high temperatures (Sharkey, 1996). Isoprene is synthesized according to the DOXP pathway (Schwender \textit{et al.}, 1997; Zeidler \textit{et al.}, 1997).

The DOXP pathway starts with the formation of 1-deoxy-d-xylulose-5-phosphate (DOXP) from glyceraldehyde-3-phosphate and pyruvate by the enzyme DOXP synthase (see Lichtenthaler, 1998 and references therein) (Fig. 1). DOXP is subsequently transformed to 2-C-methyl-d-erythritol 4-phosphate by an intramolecular C–C skeleton rearrangement (Fig. 1). This is indicated by the specific incorporation of \textsuperscript{13}C- or \textsuperscript{14}C-labeled 1-deoxy-d-xylulose into isoprene and phytol (Zeidler \textit{et al.}, 1997; Schwender \textit{et al.}, 1997), into \beta-carotene (Arigoni \textit{et al.}, 1997), into 2-C-methyl-d-erythritol of plants (Sagner \textit{et al.}, 1998), and into bacterial ubiquinone (Rosa Putra \textit{et al.}, 1998b). In addition, a DOXP reductoisomerase which trans-
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Pyruvate + GA-3-P

\[ \text{DOXP synthase} \]

1-Deoxy-\(\beta\)-xylulose-5-P (DOXP)

\[ \text{DOXP reductoisomerase} (+ NADPH) \]

2-C-Methyl-\(\beta\)-erythritol-4-P

Isopentenyl diphosphate (IPP)

Fig. 1. Biosynthesis of the isoprenoid precursor isopentenyl diphosphate (IPP) from pyruvate and GA-3-P via the non-mevalonate DOXP pathway. The C-atoms 1, 2 and 4 of IPP derive from GA-3-P and the C-atoms 3 and 5 from pyruvate.

forms DOXP to 2-C-methyl-\(\beta\)-erythritol-4-phosphate was cloned in \(E.\ coli\) (Takahashi et al., 1998). It appears that the DOXP reductoisomerase reaction may have a mechanism similar to the ketol acid reductoisomerase (KARI), which operates in the biosynthesis of valine, leucine and isoleucine (Aulabaugh and Schloss, 1990). Both enzymes catalyze a C=C skeleton rearrangement followed by a NADPH-dependent reduction step (Aulabaugh and Schloss, 1990; Takahashi et al., 1998).

In our search for inhibitors of the plants’ DOXP pathway we were looking for analogues to KARI inhibitors, such as N-isopropyl-N-hydroxyoxamate (IpOHA) (Aulabaugh and Schloss, 1990; Biou et al., 1997). Fosmidomycin (Fig. 2), an antibiotic produced by \(Streptomyces lavendulae\) (Okuhara et al., 1980), had formerly been shown to affect isoprenoid biosynthesis in several bacteria (Shigi, 1989) and also to have herbicidal activity (Patterson, 1987; Kamuro et al., 1988). However, its mode of action has so far not been evaluated.

For this reason we tested, if fosmidomycin can inhibit the plants’ biosynthesis of plastidic isoprenoids, such as carotenoids, phytol (side-chain of chlorophylls), and isoprene which are formed via the novel, alternative, non-mevalonate DOXP pathway of IPP biosynthesis (Lichtenthaler, 1998).

Materials and Methods

Plant material

Barley grains (\(Hordeum vulgare\) L., cv. Alexis) were germinated in the dark. Tomato plants (\(Lycoopersicon esculentum\) L., cv. Sweet 100) were grown in the greenhouse, and duckweed plantlets (\(Lemna gibba\) L.) in continuous light (100 \(\mu\)mol m\(^{-2}\)s\(^{-1}\)) on a liquid mineral medium (Lichtenthaler et al., 1997b).

Greening of etiolated barley leaves

Primary etiolated leaves of 5–6 d old barley seedlings were cut and placed with the cut ends in the dark into a mineral medium (Lichtenthaler et al., 1997b) with different concentrations of fosmidomycin. The inhibitor was taken up by transpiration which was promoted by a continuous air stream (ventilator). After a 5 h dark period for uptake of the fosmidomycin the leaves were exposed to light (100 \(\mu\)mol m\(^{-2}\)s\(^{-1}\)) for a 18 h greening period.

Ripening of tomato fruits

Small tomato fruits were harvested just at the beginning of ripening when the change of colour from green to red was first visible. Into each tomato fruit (5–6 g fresh weight) 10–30 \(\mu\)l of a 50 mM solution of fosmidomycin was injected.
control fruits with much lycopene accumulation and the treated tomato fruits were extracted after 10 d.

**Growth of duckweed plantlets**

Plantlets of *Lemna gibba* L. were grown for 6 d on a liquid mineral medium (Lichtenthaler et al., 1997b) under continuous light (100 μmol m⁻² s⁻¹) without (controls) and with fosmidomycin (10⁻⁴ M, 5×10⁻⁵ M, 2.5×10⁻⁵ M) in the nutrition solution.

**Determination of chlorophylls and carotenoids**

After grinding of plant material and cold extraction with acetone, chlorophylls *a* and *b* as well as total carotenoids *x+c* were determined spectrophotometrically (Lichtenthaler, 1987).

**Isoprene emission**

Leaves of *Populus nigra* L., *Platanus × acerifolia* and *Chelidonium majus* L. were taken from plants growing on the campus of the University of Karlsruhe. Isoprene emission was quantified using a newly developed UV-cuvette test system (Zeidler and Lichtenthaler, 1998). Freshly cut rectangular leaf pieces were placed at one inner side of a quartz cuvette containing 0.5 ml of either water or an aqueous solutions of fosmidomycin or 1-deoxy-D-xylulose. The leaf pieces were illuminated in the closed cuvette with white light (1000 μmol photons m⁻² s⁻¹) after different incubation times (1 h to 51 h) at a temperature of 25–35 °C. After the illumination (ca. 1 h) UV-spectra (200–250 nm) were measured. The isoprene concentration in the cuvette air was assessed by the absorption at the maximum at 216 nm.

**Isolation of DOXP synthase and enzyme assay from *E. coli***

The *E. coli* strain XL1-Blue harboring plasmid pTAC-ORF2, in which the 1-deoxy-D-xylulose-5-phosphate synthase gene of *E. coli* was cloned, was grown as described by Lois et al. (1998). A crude DOXP synthase extract of the *E. coli* cultures was used for the enzyme assay applying 0.34 mM [2-¹⁴C]pyruvate (15.9 mCi/mm, DuPont NEN), 20 mM GA-3-P, and crude enzyme in a concentration of 420 μg protein/ml.

The reaction proceeded at 37 °C. At the beginning and after 5 min 10 μl of the solution were taken and the reaction stopped by 10 μl ice cold acetone. 1-Deoxy-D-xylulose-5-phosphate and pyruvate were separated by TLC (n-propyl alcohol/ethylacetate/H₂O 6:1:3; Lois et al., 1998). Bands (DOXP Rf 0.35, pyruvate Rf 0.59) were scraped off and the radioactivity was counted in a liquid scintillation counter (Packard 2000 CA). Concentrations of 10⁻⁴ M and 10⁻³ M fosmidomycin were applied in the enzyme test.

**Synthesis of fosmidomycin**

Fosmidomycin, 3-(N-formyl-N-hydroxyamino)-propylphosphonate, was synthesized using a combination of the protocols reported before (see Kamiya et al., 1980; Öhler and Kanzler, 1995). Detailed protocols of the synthesis can be obtained from one of the authors, H. Jomaa, Giessen.

**Results and Discussion**

Since many algae possess the DOXP pathway for the synthesis of plastidic isoprenoid pigments (Schwender et al., 1997; Disch et al., 1998), we tested the effect of fosmidomycin in a red alga. When applied to *Cyanidium caldarium* fosmidomycin fully inhibited cell multiplication and pigment accumulation at a level of 5×10⁻⁵ M and 5×10⁻⁴ M (data not shown). Also in higher plants the formation of chlorophylls and carotenoids is blocked by fosmidomycin as shown below.

Ripening tomatoes produce the red carotenoid lycopene which is formed via the DOXP pathway (Schwender and Lichtenthaler, 1998). When fosmidomycin was injected into green ripening tomato fruits, the accumulation of this secondary carotenoid lycopene (in controls 700 μg per g dw) within 10 days was inhibited to about 60–70%.

In already greened primary barley leaves fosmidomycin had no effect on the level of chlorophylls and carotenoids, suggesting that the substance does not induce or promote a pigment breakdown. It is known that etiolates leaves accumulate in the dark carotenoids and upon a short illumination contain some chlorophyll coming from phototransformation of protochlorophyllide (Lichtenthaler, 1969; Babani and Lichtenthaler, 1996). With the light-stimulated greening and thylakoid formation an enhanced accumulation of chloro-
phylls and carotenoids takes place. When 6 d old dark grown etiolated primary barley leaves, which had taken up fosmidomycin, were placed into continuous light, the light-induced formation of carotenoids and chlorophylls was, however, strongly inhibited (Table I). Similar inhibition results were obtained with leaves of 4 d and 5 d old etiolated barley seedlings. The inhibitory effect was also measurable at $10^{-5}$ M fosmidomycin, but barely detectable when placing the cut leaves into a $10^{-6}$ M solution of fosmidomycin.

The accumulation of newly synthesized carotenoids in barley leaves was inhibited by fosmidomycin to a higher degree than that of newly formed chlorophylls which is also seen in considerably increasing values of the pigment ratio $(a+b)/(x+c)$ of the newly formed pigments (Table I). During greening of etiolated barley leaves, the ratio chlorophyll $a/b$ decreases from initially high values (about 20) to the ratio of about 3 in fully developed green leaves (Lichtenthaler, 1969; Babani and Lichtenthaler, 1996). In contrast, fosmidomycin-treated etiolated barley leaves exhibited a dose-dependent increase in the chlorophyll $a/b$ ratio (Table I) indicating that chlorophyll $b$ accumulation is inhibited to a higher degree than that of chlorophyll $a$. The matter is that in etiolated, fosmidomycin-treated leaves the whole greening process is blocked, and consequently the inhibition effect appears to be stronger in those isoprenoid components which, during greening, accumulate later than others, such as chlorophyll $b$ after chlorophyll $a$, and newly synthesized carotenoids after chlorophylls. This is to be expected when fosmidomycin blocks the plastidic DOXP pathway of IPP formation, and as a consequence also the formation of chlorophylls (phytyl side chain) and carotenoids, which are major constituents of functional and photochemically active thylakoid membranes.

The accumulation of prenyl pigments in duckweed plantlets (*Lemna gibba*) growing in liquid media with different concentrations of fosmidomycin was also affected. The visual appearance of the plantlets, cultivated on fosmidomycin, suggested that in the new developed pale green leaves the pigment formation was strongly inhibited, whereas the pigment content of the green starting leaves was maintained. The fosmidomycin-treated *Lemna* plantlets exhibited a slightly higher chloro-

### Table I. Inhibition by fosmidomycin of *de novo* biosynthesis and accumulation of carotenoids and chlorophylls during greening of illuminated etiolated primary leaves of barley during an 18 h light period.

25 primary etiolated leaves of 6 d old barley seedlings were placed with their cut ends in the dark into a mineral medium (Schwender et al., 1997) with addition of fosmidomycin at different concentrations. The initial chlorophyll $(a+b)$ and carotenoid $(x+c)$ content of the etiolated leaves after a short light exposure (<5 min) was 253 and 726 µg per g dry weight (dw), respectively. The percentage of inhibition of isoprenoid pigment accumulation is given in parentheses. The pigment content and ratios shown are those of the newly accumulated pigments in the light.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Chlorophyll $a+b$ (µg per g dw)</th>
<th>Carotenoids $x+c$ (µg per g dw)</th>
<th>Pigment ratios $(a+b)/(x+c)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4361 (0%)</td>
<td>603 (0%)</td>
<td>3.65 7.2</td>
</tr>
<tr>
<td>Fosmidomycin</td>
<td>$10^{-6}$ M</td>
<td>4201 (4%)</td>
<td>552 (8%)</td>
</tr>
<tr>
<td></td>
<td>$10^{-5}$ M</td>
<td>2747 (37%)</td>
<td>179 (70%)</td>
</tr>
<tr>
<td></td>
<td>$10^{-4}$ M</td>
<td>428 (91%)</td>
<td>0 (100%)</td>
</tr>
</tbody>
</table>

### Table II. Inhibition of chlorophyll and carotenoid accumulation in *Lemna* plantlets after growth on fosmidomycin.

Single duckweed plantlets (*Lemna gibba*) with 3 to 4 green leaves were grown in multiwell plates (1 ml) for 6 d on a mineral medium in the presence of fosmidomycin (white light, 100 µmol photons m$^{-2}$s$^{-1}$). In the control and $2.5 \times 10^{-5}$ M setups each plantlet divided in average to 7 or 8 plantlets, respectively. Mean of 8 separate extractions ± SD. *p < 0.005. The percentage inhibition is given in parentheses.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Chlorophyll $a+b$ (µg per 10 plantlets)</th>
<th>Carotenoids $x+c$ (µg per 10 plantlets)</th>
<th>Pigment ratios $(a+b)/(x+c)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>34.9 ± 7.0 (0%)</td>
<td>9.5 ± 1.8 (0%)</td>
<td>3.03 3.67</td>
</tr>
<tr>
<td>$2.5 \times 10^{-5}$ M</td>
<td>23.0 ± 3.4* (34%)</td>
<td>6.4 ± 1.0* (33%)</td>
<td>3.31 3.59</td>
</tr>
</tbody>
</table>
phyll \(a/b\) ratio, whereas the pigment ratio \((a+b)/(x+c)\) remained unchanged (Table II).

The observed inhibitory effects of fosmidomycin on carotenoid biosynthesis in ripening tomato fruits, on carotenoid and chlorophyll formation in illuminated etiolated barley shoots, and in duckweed plantlets suggest that fosmidomycin is an efficient inhibitor of the plant's DOXP pathway, which is known to be responsible for the biosynthesis of these isoprenoid pigments.

Fosmidomycin also efficiently inhibited the emission of isoprene from leaf pieces of *Populus nigra*, *Platanus × acerifolia*, and *Chelidonium majus* in a dose-dependent manner (Table III and Fig. 3). The \(I_{50}\) values for 50% inhibition of the isoprene emission lie in the range of 0.5–1.0×10\(^{-6}\) m. The inhibition of isoprene emission (at 5×10\(^{-6}\) m) could be overcome by addition of high levels (3×10\(^{-2}\) m) of 1-deoxy-d-xylulose (DOX), which is an effective precursor of the DOXP pathway of IPP biosynthesis. This observation can be explained assuming that fosmidomycin is a competitive inhibitor of the 1-deoxy-d-xylulose-5-phosphate reductoisomerase, the enzyme that follows the DOXP synthase in the DOXP pathway of IPP formation (Fig. 1).

In *Platanus* leaf disks floating at first on a DOX solution (6 mm), DOX was taken up into the leaf as shown by TLC of leaf extracts, but later metabolized, when placing the leaf disks on water, most probably to isoprene under the given conditions of high light and high temperature. DOX is efficiently incorporated into isoprene emitted by *Platanus* (Zeidler and Lichtenthaler, 1998) and other

Table III. Inhibition of isoprene emission of leaf pieces by different concentrations of fosmidomycin at different incubation times.

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Concentration [m]</th>
<th>Incubation time</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Populus nigra</em></td>
<td>5×10(^{-4})</td>
<td>20 h</td>
<td>93</td>
</tr>
<tr>
<td><em>Chelidonium majus</em></td>
<td>5×10(^{-7})</td>
<td>14 h</td>
<td>60</td>
</tr>
<tr>
<td><em>Platanus × acerifolia</em></td>
<td>5×10(^{-6})</td>
<td>51 h</td>
<td>79</td>
</tr>
<tr>
<td><em>Platanus × acerifolia</em></td>
<td>3.2×10(^{-6})</td>
<td>39 h</td>
<td>84</td>
</tr>
<tr>
<td><em>Platanus × acerifolia</em></td>
<td>1.6×10(^{-6})</td>
<td>39 h</td>
<td>55</td>
</tr>
</tbody>
</table>

In further experiments we could show in enzyme preparations of recombinant *E. coli* DOXP synthase that fosmidomycin does not inhibit the first enzyme of the DOXP pathway, i.e. the DOXP synthase (Fig. 1). Concentrations of 10\(^{-4}\) m and even 10\(^{-3}\) m fosmidomycin had no inhibitory effect on the activity of the DOXP synthase.

All results mentioned here, such as inhibition of carotenoid, chlorophyll and isoprene biosynthesis
by fosmidomycin, non-metabolism of DOX in leaf disks in the presence of fosmidomycin, as well as non-inhibition of the DOXP synthase by fosmidomycin are in agreement with the view that the target of fosmidomycin lies in the DOXP pathway in the enzymatic step following the DOXP synthase. The compensation of the fosmidomycin inhibition by high doses of DOX can be taken as an indication that the DOXP reductoisomerase is the target enzyme of fosmidomycin. Fosmidomycin has a structural similarity to IpOHA (cf. Fig. 4). Both, IpOHA and fosmidomycin, are analogous to the rearrangement products of the enzymes KARI and DOXP reductoisomerase, respectively (cf. Fig. 4). It appears that fosmidomycin is a competitive inhibitor and may bind to the DOXP reductoisomerase in a similar way as its natural substrate DOXP. The binding of fosmidomycin to its putative target enzyme may be analogous to the binding of IpOHA to KARI in the biosynthesis of valine, leucine or isoleucine (Fig. 4). Studies to clone and express plant DOXP reductoisomerases have been started in order to further characterize the target site of fosmidomycin.

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