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Fosmidomycin, a Specific Inhibitor of 1-Deoxy-D-Xylulose 5-Phosphate Reductoisomerase in the Nonmevalonate Pathway for Terpenoid Biosynthesis

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Abstract.
Fosmidomycin inhibited 1-deoxy-D-xylulose 5-phosphate reductoisomerase in an alternative nonmevalonate pathway for terpenoid biosynthesis with IC_{50} of 8.2 nM.

Keywords: Inhibitor, Fosmidomycin, 2-*C*-methylerythrose 4-phosphate, 1-Deoxy-D-xylulose 5-phosphate reductoisomerase

Since the initial discovery of the mevalonate pathway, it was widely accepted that isopentenyl diphosphate (IPP), a fundamental unit in terpenoid biosynthesis, was only formed through the ubiquitous mevalonate pathway. However, it has been disclosed recently that organisms including several bacteria, green algae and chloroplasts of higher plants use an alternative mevalonate-independent pathway (nonmevalonate pathway) for the formation of IPP.[1-12]

Recent studies revealed that the initial step of this pathway is the formation of 1-deoxy-D-xylulose 4-phosphate **1** (DXP) by condensation of pyruvate and glyceraldehyde 3-phosphate (Fig. 1A).[5] In the second step the intramolecular rearrangement of **1** was assumed to give a hypothetical rearrangement intermediate 2-*C*-methylerythrose 4-phosphate **2** which was then converted to 2-*C*-methyl-D-erythritol 4-phosphate **3** by an unspecified reduction process.[6] More recently, we have succeeded in the cloning[13,14] and overexpression of the gene encoding DXP reductoisomerase from *Escherichia coli*. This new enzyme catalyzed the formation of **3** in the presence of NADPH in a single step (Fig. 1A), and our attempts to detect the formation of **2** as the reaction intermediate were totally unsuccessful.

The following reactions leading from **3** to IPP, however, have not been described to date.

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In addition, distribution of the nonmevalonate pathway among living organisms has remained as an intriguing and important problem.

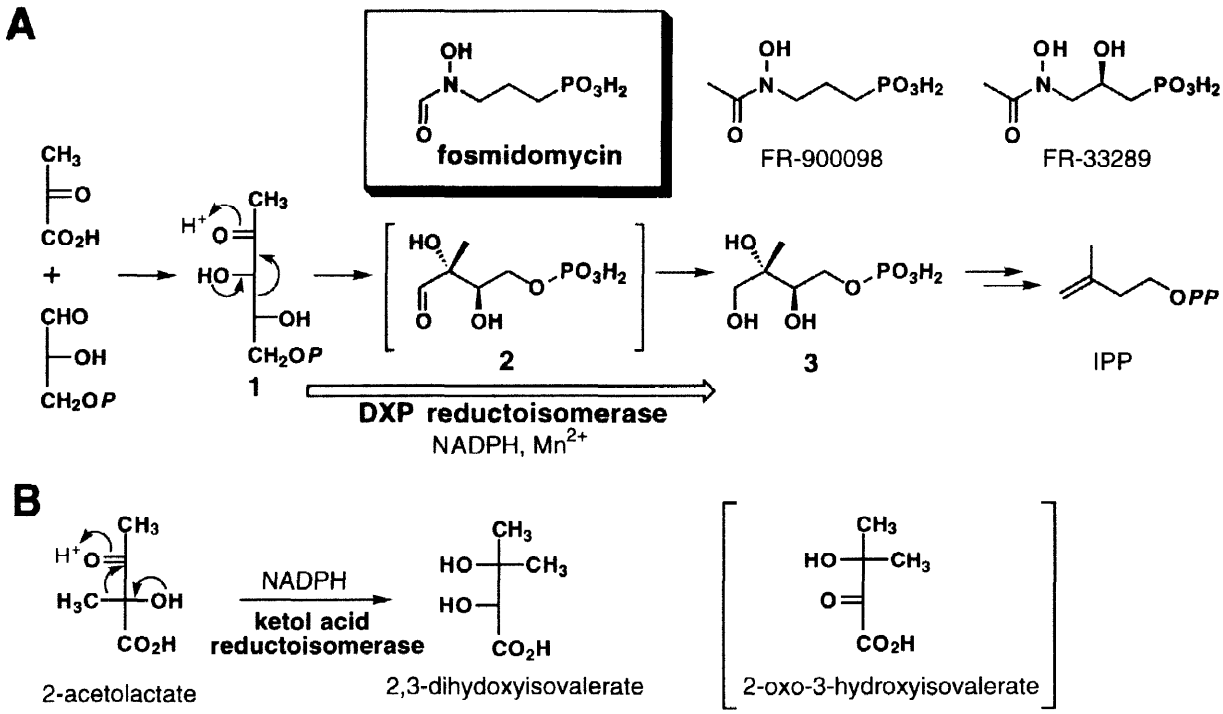
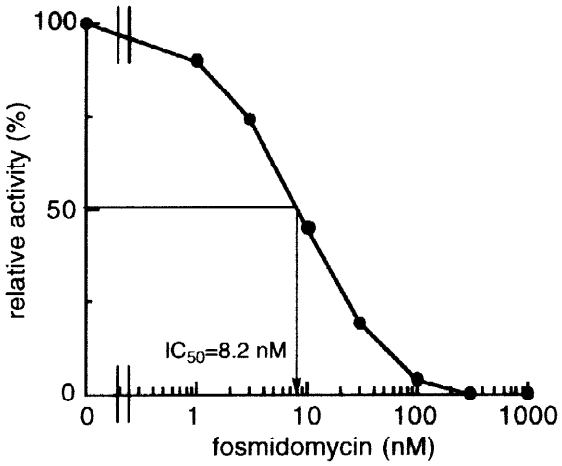


Figure 1. (A) Alternative nonmevalonate pathway for IPP biosynthesis and structures of fosmidomycin, FR-900098 and FR-33289. (B) Ketol acid reductoisomerase reaction.

Since specific inhibitors of the nonmevalonate pathway were expected to be useful tools to address these unsolved issues, we attempted by database search to find antibiotics active against *E. coli*[15] and *Bacillus subtilis*[15] with the nonmevalonate pathway, but inactive against *Staphylococcus aureus*[16] possessing the mevalonate pathway with an expectation that such inhibitors might have already been reported as antibacterial substances. As a result, fosmidomycin (FR-31564) showing the expected antibacterial spectrum came up as a candidate for an inhibitor of the nonmevalonate pathway.

Fosmidomycin (3-(*N*-formyl-*N*-hydroxyamino)propylphosphonic acid) has a potent antibacterial activity against most Gram-negative and some Gram-positive bacteria.[17-19] Due to the inhibition of both menaquinone and carotenoid biosynthesis in *Micrococcus luteus* by the antibiotic, it had been proposed that the lethal effect of the antibiotic lied in the biosynthetic pathway for terpenoids.[20] From these pioneering studies on fosmidomycin, we assumed that the antibiotic may inhibit DXP reductoisomerase in the nonmevalonate pathway.

In order to evaluate the activity of fosmidomycin, we used the purified recombinant *E. coli* DXP reductoisomerase which was prepared as described previously.[13,14] The DXP reductoisomerase assay system consisted of 100 mM Tris-HCl (pH 7.5), 1 mM $MnCl_2$, 0.3 mM NADPH and 0.3 mM enzymatically synthesized **1**[13,14] in a final volume of 200 μl. The reaction was initiated by adding the enzyme solution to the complete assay mixture. The oxidation of



NADPH was monitored at 340 nm with a Benchmark Microplate Reader (BIORAD) adjusted at 37 °C.

As expected, fosmidomycin strongly inhibited the DXP reductoisomerase activity in the dose dependent manner with an IC_{50} value being 8.2 nM (Fig. 2). Lineweaver-Burk plot indicated mixed (competitive and noncompetitive) inhibition with a *Ki* value of 38 nM.[21] Therefore, fosmidomycin is a mixed-type inhibitor of DXP reductoisomerase.

Figure 2. Inhibition of the DXP reductoisomerase activity by fosmidomycin.
Data are expressed relative to the enzyme activity (9.2 U/mg protein)[22] without fosmidomycin.

We next investigated the antibacterial activity of fosmidomycin against *E. coli*. The growth was completely inhibited by 3.13 μg/ml fosmidomycin. However, the inhibitory effect of the antibiotic on the growth of *E. coli* was suppressed by addition of 0.025 % 2-*C*-methylerythritol,[23] the free alcohol of **3**, into the growth medium. In addition, fosmidomycin had no effect on the growth of an *E. coli* DXP reductoisomerase disruptant[24] in the presence of 0.025 % 2-*C*-methylerythritol. No effect of fosmidomycin on the disruptant is interpreted by the lack of the molecular target in the disruptant. From these results, fosmidomycin is concluded to be a specific inhibitor of DXP reductoisomerase in the nonmevalonate pathway.

It should be emphasized that fosmidomycin and **2**, a hypothetical rearrangement intermediate of DXP reductoisomerase (Fig. 1A), have close structural similarities with each other in that they possess the formyl and phosphonate (or phosphate) functions separated by five chemical bonds. It is interesting to note that fosmidomycin was first prepared by synthesis during chemical optimization of structurally related FR-33289 and FR-900098[25] with an *N*-acetyl group and that the antibiotic was later detected as a minor product of *Streptomyces lavendulae*.

The DXP reductoisomerase reaction proceeds presumably in a similar manner to the ketol acid reductoisomerase reaction (Fig. 1B). Interestingly 2-oxo-3-hydroxyisovalerate, a compound with a carbonyl function, which is estimated to be an analog of a hypothetical biosynthetic intermediate **2**, inhibited ketol acid reductoisomerase.[25] This fact may support that fosmidomycin inhibits DXP reductoisomerase as an analog of **2**. If so, fosmidomycin and its structural analogs such as FR-33289 and FR-900098 may be useful tools for elucidating the detailed reaction mechanism of DXP reductoisomerase.

As mentioned above, very little is known about the distribution of the nonmevalonate pathway in bacteria. This was due to the lack of reliable and convenient analytical methods to detect the presence of the nonmevalonate pathway in microorganisms. With fosmidomycin in

hand, it has become possible to classify efficiently the microorganisms into two groups, one using the mevalonate pathway and the other using the nonmevalonate. This classification now in progress in our laboratory is very important from not only biological but also pharmaceutical views, because DXP reductoisomerase could be a new molecular target for chemotherapeutically useful drugs.[27]

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