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On the Reduction Steps in the Mevalonate Independent 2-*C*-Methyl-D-erythritol 4-phosphate (MEP) Pathway for Isoprenoid Biosynthesis in the Bacterium *Zymomonas mobilis*

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Abstract. *Zymomonas mobilis* synthesizes triterpenoids of the hopane series *via* the methylerythritol phosphate (MEP) pathway. Owing to the peculiar metabolic pathways in this bacterium, [1-^{2}H]glucose is the appropriate substrate for generating *in vivo* deuterium labeled NADP^{2}H. After feeding of *Z. mobilis* with [1-^{2}H]glucose, deuterium labeling was found on all carbon atoms derived from C-2 and C-4 of isopentenyl diphosphate. The deuterium at C-4 arose from the NADPH dependent reduction catalyzed by the reducto-isomerase converting 1-deoxy-D-xylulose 5-phosphate into 2-*C*-methyl-D-erythritol 4-phosphate. The deuterium at C-2 resulted from an additional reduction at a yet undetermined steps of the MEP biosynthetic pathway.

Bacterial triterpenoids of the hopane series[1] played a key role in the discovery and the elucidation of the alternative mevalonate-independent route for isoprenoid biosynthesis (Scheme 1).[2] This metabolic route was found in bacteria, algae and plant plastids.[3] Pyruvate **1** and glyceraldehyde 3-phosphate **2** (GAP) are the first precursors.[2d] A condensation catalyzed by a thiamin diphosphate dependent synthase yields 1-deoxy-D-xylulose 5-phosphate **3** (DXP).[4] A reducto-isomerase catalyzes the next step: the rearrangement of the straight chain of DXP and the concomitant NADPH dependent reduction of the resulting aldehyde into 2-*C*-methyl-D-erythritol 4-phosphate **5** (MEP).[5] Feeding experiments performed with ^{2}H or ^{13}C labeled deoxyxylulose showed that the free pentulose, or rather its 5-phosphate, was not only the precursor for isoprenoids,[6] but also for thiamin diphosphate[7] and pyridoxol phosphate.[8] The formation of DXP does not accordingly represent the committed step in the non-mevalonate pathway. MEP, in contrast, seems devoid of any other role. Its biosynthesis might represent the key step of the mevalonate-independent route. Although free methylerythritol was incorporated into the isoprenoids of *Escherichia coli*,[9] no data is available on other intermediates and reactions implied in the conversion of MEP into isopentenyl diphosphate **6** (IPP). Formally, the reaction sequence from MEP to IPP requires at least one phosphorylation, two reductions and the elimination of two molecules of water.

Hopanoids **10** and **11** (Scheme 1) are synthesized via the mevalonate-independent route in *Zymomonas mobilis*.[2c] This bacterium has no complete tricarboxylic acid cycle.[10] NADH and consequently NADPH, the cofactors involved in most biological reductions, are obtained either from the oxidation of glucose 6-phosphate into gluconate 6-phosphate lactone by the glucose 6-phosphate dehydrogenase or of GAP into 3-phosphoglyceric acid by the GAP dehydrogenase. In addition, glucose is metabolized via the Entner-Doudoroff pathway, and the resulting pyruvate is not converted into GAP because of the irreversibility of the reaction catalyzed by the enolase of this bacterium. Such a glucose catabolism implies that C-1 of glucose is completely lost as carbon dioxide by the decarboxylation of pyruvate and is not incorporated into the isoprenoids of *Z. mobilis*.[2c] When, however, [1-^{2}H]glucose is utilized as sole carbon source, [4*R*-^{2}H]- and [4*S*-^{2}H]NADPH are formed by the action of the glucose 6-phosphate dehydrogenase, the GAP dehydrogenase and the two zinc or iron dependent alcohol dehydrogenases converting acetaldehyde into ethanol.[11] This makes

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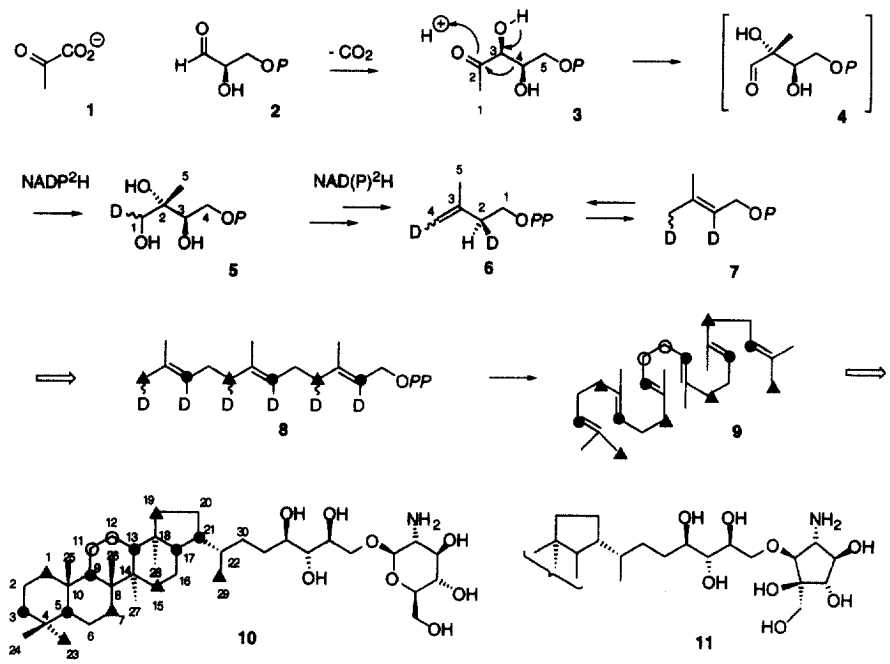
[1-^{2}H]glucose a well suited substrate for studying the reduction steps of the MEP route in *Z. mobilis*. The bacterium was grown in the presence of [1-^{2}H]glucose,[12] and the hepta-acetates of the two major bacteriohopanetetrol derivatives **10** and **11** were isolated[2c] and analyzed by ^{1}H-NMR and ^{13}C-NMR for deuterium induced α-, β- and γ-shifts.[13,14] Analysis of the labeling pattern was facilitated by the hopane skeleton. Indeed, it results from the cyclization of squalene **9** which is formed from two equivalent moieties derived from farnesyl diphosphate **8** and consequently symmetrical.



Scheme 1. Hopanoid biosynthesis in *Zymomonas mobilis via* the mevalonate-independent MEP pathway. Deuterium labeling introduced by (▲) the deoxyxylulose reducto-isomerase, (●) an unknown enzyme involved in the formation of IPP, (○) the squalene synthase.

The NMR data of the hepta-acetates of hopanoids **10** and **11** was in accord with the presence of one deuterium on all carbon atoms derived from C-2 and C-4 of IPP with a ca. 50% isotopic abundance, as deduced from the integration of the signals from the C-23 and C-29 methyl groups in ^{1}H-NMR and the relative intensities of the ^{13}C signals presenting β-shifts.[14] Direct evidence for the presence of a deuterium was obtained for the C-23 and C-29 methyl groups. In ^{1}H-NMR, the non-labeled methyl signal was accompanied by an upfield shifted -CH_2D signal,[14a] and, in the ^{13}C-NMR spectrum, the singlet, corresponding to the unlabeled methyl group, was accompanied by a triplet from the -CH_2D group characterized by an upfield α-shift and a $^1J_{^{13}C,D}$ coupling constant.[14c] Similar α-shifts were observed for the signals of C-1, C-7 and C-19, confirming directly the presence of one deuterium on these carbon atoms. In the ^{13}C-NMR spectrum, nearly all carbon atoms corresponding to C-1 and C-3 of IPP were characterized by three signals in a 30:54:16 ratio: a singlet corresponding to no labeling on the two adjacent carbon atoms was accompanied by two additional singlets, corresponding to β-shifts induced either by the presence of a single deuterium on one of the adjacent carbon atoms derived from C-2 or C-4 of IPP, or by the simultaneous presence of a deuterium on each of these adjacent carbon atoms. For the C-10 and C-18 positions with three possible adjacent deuteriums, only two additional signals were found, the probability that the three adjacent positions were simultaneously labeled being too low. β-Shifts were expected for C-12 and C-17, because of the presence of deuterium at respectively C-13 and C-21, but were not observed. The presence of two an additional deuterium was also detected at C-11 and C-12. Indeed, the signals of C-9 and C-13 showed β-shifts induced by the deuterium atom introduced

during the reduction step catalyzed by the squalene synthase after the condensation of two farnesyl diphosphate moieties **8**. As squalene **9** is symmetrical, this deuterium is present with the same probability at C-11 and C-12 on the hopane skeleton, and the intensity of the β-shifted signals was consequently lower for C-9 and C-13 than those of the signals described above. The numerous γ-shifts were in accord with the proposed localization of deuterium labeling.

All isoprenic units were identically labeled in the hopanoids. No difference was observed, whatever they were derived from IPP **6** or from dimethylallyl diphosphate **7** (DMAPP). The deuterium found on the carbon atoms derived from C-4 of IPP, corresponding to C-1 of MEP, was introduced by the DXP reducto-isomerase: this enzyme isomerizes DXP **3** into 2-*C*-methyl-D-erythrose 4-phosphate **4** which is concomitantly reduced by NADPH.[5] Presence of deuterium on the carbon atoms derived from C-4 of IPP confirmed that the catabolism of [1-^{2}H]glucose allowed deuterium labeling of NADPH in *Z. mobilis*. The hydrogen atom found in isoprenic units on the carbon atoms derived from C-2 of IPP or DMAPP does not derive from DXP or from MEP. It is introduced at a later step, which has still to be identified, *via* a NADH or NADPH dependent reduction. These results fitted perfectly with those obtained after incubation of 1-deoxy-D-[2-^{13}C, 4-^{2}H]xylulose with a cell culture of *Catharanthus roseus*.[15] The deuterium was lost in all isoprenic units from phytol and from lutein, which are synthesized *via* the MEP pathway. In contrast, the incubation of the [4-^{2}H]deoxyxylulose with *E. coli* showed two different labeling patterns for the isoprenic units from the prenyl side-chain of ubiquinone.[16] Those derived from IPP were not labeled, whereas the terminal starter unit derived from DMAPP was characterized by a full retention of deuterium. No satisfactory explanation was proposed for this contradiction at the present stage of the elucidation of the metabolic route.

A similar deuterium incorporation was reported for the biosynthesis of sesquiterpenoids of the pentalenolactone series in *Streptomyces* UC5319, which are synthesized *via* the MEP pathway.[17] Distribution of the deuterium after feeding of [6,6-$^2H_2$]glucose showed a striking pattern: it was in contradiction with the MVA pathway, but fitted with the presence of reductions at the sites described above for the MEP pathway in *Z. mobilis*. *Streptomyces* utilizes glucose via glycolysis. Through the action of the aldolase and the triose phosphate isomerase, [6,6-$^2H_2$]glucose is converted into [1-^{2}H]glucose, yielding finally pools of deuterium labeled NADH and NADPH. As observed in the case of the hopanoids of *Z. mobilis*, deuterium labeling was observed on all carbon atoms of the pentalenene derivatives derived from C-4 of IPP as well as on C-1 of pentalenolactone E corresponding to C-2 of IPP. All other carbon atoms derived from C-2 of IPP lost their deuterium labeling in the in pentalenolactone E or in other pentalenene derivatives by later oxidation steps of this biosynthetic pathway.

Experimental results indicated that IPP is not synthesized from DMAPP in *Catharanthus roseus* cell cultures,[15] and that IPP, and not DMAPP, might directly result in peppermint from the MEP pathway.[18] The IPP isomerase and the prenyl transferase present in *E. coli* the same enantioselectivity as the corresponding yeast enzymes, both eliminating the pro-*R* hydrogen of C-2 from IPP.[19] Assuming that the IPP isomerase and the prenyl transferases possess the same stereoselectivity in *Z. mobilis* and in *E. coli* , the retention of a deuterium in the isoprenic units of the *Z. mobilis* hopanoids after feeding of [1-^{2}H]glucose suggested that the pro-*R* hydrogen of unknown origin at C-2 of IPP was removed, and consequently that the hydride labeled from [1-^{2}H]glucose and introduced by reduction was in the pro-*S* position (Scheme 1).

Growth of *Z. mobilis* on [1-^{2}H]glucose confirmed the presence in this bacterium of a reduction site on carbon atoms corresponding to C-4 of IPP: this was the signature of the DXP reducto-isomerase and indicated that deuterium labeled NADPH was synthesized from the glucose isotopomer. It also pointed out two other features. The hydrogen at C-4 of DXP or at C-3 of MEP is lost when these precursors are incorporated into the hopanoids, and the hydrogen found on all carbon atoms corresponding to C-2 of IPP is introduced by a NADH or NADPH dependent reduction. This hydrogen loss and this reduction occur at yet unidentified steps of the MEP pathway and represent a matter of further investigations. Labeling of the bacteriohopane side-chain yielded interesting clues on its biosynthesis and will be discussed elsewhere.

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12. *Zymomonas mobilis* (ZM6, DSM 3580, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, FRG) was grown at 30°C for 20 h in anaerobic conditions on a synthetic medium (2 l, pH 5) containing [1-^{2}H]glucose (10 g l^{-1}, isotopic abundance 99%, Omicron Biochemicals Inc., South Bend, Indiana), KH_2PO_4 (3.5 g l^{-1}), $MgSO_4.7H_2O$ (0.5 g l^{-1}), NH_4Cl (1.6 g l^{-1}), $(NH_4)_2Fe(SO_4)_2.6H_2O$ (14 mg l^{-1}), citric acid (0.2 g l^{-1}), biotin (1 mg l^{-1}), calcium panthotenate (1 mg l^{-1}). The heptaacetates of hopanoids (**10**) (7.5 mg) and (**11**) (4.9 mg) were isolated from the lyophilized biomass (490 mg) as previously reported.[2c]
13. NMR spectra were recorded in $CDCl_3$ solution at 300 K on a Bruker ARX 500 spectrometer equipped with a Silicon Graphics station using $CDCl_3$ (δ=7.260 ppm) or $^{13}CDCl_3$ (δ=77.030 ppm) as internal standard. ^{13}C-NMR spectra were measured as follows: 25° pulses (4 μsec); repetition time: 1.12 sec; spectra width: 26.3 kHz; data set: 32 kilo-words; zero filling to 34 kilo-words before Fourrier transformation; 1Hz experimental multiplication apodization; ^{1}H decoupling by WALTZ 16 during acquisition and relaxation.
14. Chemical shifts in the ^{1}H- and ^{13}C-NMR spectra and ^{2}H induced shifts in the ^{13}C-NMR spectrum were nearly identical for the hopane moiety in the spectra of the hepta-acetates of **10** and **11**. For the sake of clarity, only the part of the spectrum concerning the triterpenic moiety of acetylated **10** is described. Data concerning the side chain will be reported elsewhere.
^{1}H-NMR (500 MHz): δ (ppm) = 0.674 (3H, s, 18α-CH_3), 0.783 (3H, s, 4β-CH_3), 0.806 (3H, s, 10β-CH_3), 0.820 (1/2x2H, s, 4α-CH_2D), 0.840 (1/2x3H, s, 4α-CH_3), 0.868 (1/2x2H, d, J=6.0 Hz, 22-CH_2D), 0.892 (1/2x3H, d, J=6.5 Hz, 22-CH_3), 0.937 (6H, s, 8β- and 14α-CH_3). ^{2}H-NMR (76.7 MHz): δ (ppm) = 0.89 (4α- and 22-CH_3), 1.20-1.85 (hopane skeleton).
^{13}C-NMR (126 MHz): δ (ppm)/^{2}H induced shift (ppb) = C-1 (40.346/α-shift: -393, t, 20Hz), C-2 and C-6, (18.697/β-shifts: -105 and -198, biogenetically equivalent, both corresponding to C-1 of IPP), C-3 (42.086, broad signal/γ-shifts: -14 and -32), C-4 (33.257/β-shifts: -77 and -160), C-5 (56.084, broad signal due to up to 5 γ-shifts, no detectable α-shift), C-7 (33.305/α-shift: -364, t, J=20 Hz), C-8* (41.818/β-shifts: -89 and -165), C-9 (50.461/β-shift: -38), C-10 (37.408/β-shifts: -93 and -179), C-11 (20.974, possible shifted signal concealed by those of the acetoxy methyl groups), C-12 (23.985), C-13 (49.337/β-shift: -45), C-14* (41.700/β-shifts: -87 and -163), C-15 (33.708/possible α-shifted signal concealed by the signals of C-7, C-4 and C-23), C-16 (22.784/β-shifts: -83 and -188), C-17 (54.408, broadening of the peak, no measurable β-shift), C-18 (44.382/β-shifts: -80 and -169), C-19 (41.569/α-shift: -390, t, J=20 Hz), C-20 (27.603/β-shifts: -93 and -198), C-21 (45.946/no detectable α- or β-shifts, γ-shift: -16), C-22 (36.150/β-shifts: -70 and 141) C-23 (33.411/α-shift: -313,t, J=20 Hz), C-24 (21.593/γ-shifts: -26 and -45), C-25 (15.910/γ-shifts: -35 and -48), C-26° (16.615/γ-shifts:-13 and -29), C-27° (16.536/γ-shifts: -16 and -29), C-28 (15.875/γ-shifts: -29 and -42), C-29 (19.860/α-shift: -300, t, J=19 Hz), C-30 (30.767/γ-shift: -16). Assignments of signals bearing the same superscript may be interchanged.
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