Incorporation of [2,3-$^{13}C_2$]- and [2,4-$^{13}C_2$]-D-1-Deoxyxylulose into Ubiquinone of *Escherichia coli* via the Mevalonate-Independent Pathway for Isoprenoid Biosynthesis

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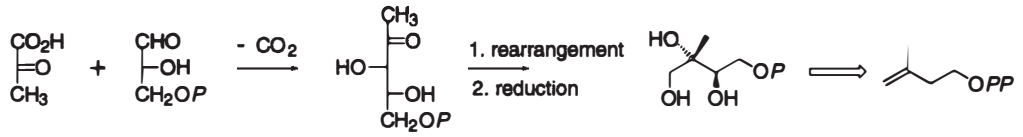
Abstract. [2,3-$^{13}C_2$]- and [2,4-$^{13}C_2$]-D-1-deoxyxylulose were synthesized from [2-^{13}C]pyruvate and [1-^{13}C]- or [2-^{13}C]-DL-glyceraldehyde using the enzyme D-1-deoxyxylulose 5-phosphate synthase from *Escherichia coli* which was overexpressed in this bacterium. These doubly-labeled isoprenoid precursors in the mevalonate independent route were incorporated into the ubiquinone of *E. coli*. ^1J $^{13}C/^{13}C$ coupling constants were respectively found in isoprenic units between carbon atoms derived from C-3 and C-4 of isopentenyl diphosphate using the former labeled precursor or between C-3 and C-2 using the latter, indicating that D-1-deoxyxylulose was incorporated without prior degradation into isoprenoids and confirming that the branched isoprenic skeleton resulted from a rearrangement of the straight chain from carbohydrate precursor.

An alternative mevalonate-independent route to isopentenyl diphosphate (Figure 1) was recently detected in many bacteria[1a-c] and several green algae.[2] It is most probably ubiquitous in higher plants where a clear-cut dichotomy was observed for isoprenoid biosynthesis. Sterols and sesquiterpenoids are synthesized in the cytoplasm via the classical mevalonate pathway whereas chloroplast-related isoprenoids seem to be mainly derived from the overlooked bacterial mevalonate-independent route.[3a-f]



1 2 3 4 5

Figure 1. Hypothetical biogenetic scheme for the mevalonate-independent route for isoprenoid biosynthesis

Pyruvate **1** and D-glyceraldehyde 3-phosphate **2** are the first precursors of this novel metabolic route.[1c] The first step is a condensation of a C_2 subunit derived from pyruvate decarboxylation, most probably hydroxyethylthiamine, on the carbonyl group of glyceraldehyde 3-phosphate. This reaction resembles that catalyzed by transketolases and yields a straight-chain C_5 carbohydrate, 1-deoxyxylulose 5-phosphate **3**. The branched isoprenic skeleton results from an enzyme catalyzed acetoin rearrangement yielding a 2*C*-methyl-D-erythritol derivative **4**. It is most probably similar to the reaction involved in L-valine biosynthesis and has been clearly identified by the $^{13}C/^{13}C$ coupling patterns resulting from the incorporation of [4,5-$^{13}C_2$]- or [U-$^{13}C_6$]glucose into the triterpenoids of the hopane series or ubiquinone from *Methylobacterium fujisawaense*, *Escherichia coli* and *Zymomonas mobilis*.[1b,c] The key role of D-1-deoxyxylulose as the first C_5 intermediate in

the mevalonate-independent pathway was demonstrated by Broers and Arigoni.[4] Indeed, addition of D-1-deoxyxylulose to the culture medium of *E. coli* increased significantly by a factor 3 to 4 the intracellular ubiquinone and menaquinone amounts. Feeding this bacterium with [1-^{2}H]- or [5,5-$^{2}H_2$]-D-deoxyxylulose resulted in the incorporation of one or two deuterium atoms on carbon atoms of the quinone prenyl side-chains respectively derived from C-1 or C-5 of isopentenyl diphosphate **5** (IPP). The same group also shed light on the role of D-1-deoxyxylulose in plant isoprenoid biosynthesis. The deuterium labeled samples were efficiently (90%) incorporated into ferruginol acetate, a diterpenoid of *Salvia miltiorrhiza*, synthesized via the mevalonate-independent route and only weakly (10%) into sitosterol mainly produced via the mevalonate pathway.[4] We report here the first attempts to enzymatically synthesize doubly-labeled [2,3-$^{13}C_2$]- and [2,4-$^{13}C_2$]-D-1-deoxyxylulose. Incorporation of such a doubly-labeled precursor into bacterial isoprenoids should tell us after examination of the $^{13}C/^{13}C$ coupling pattern in the ^{13}C NMR spectra of isoprenoids whether the five carbon atoms of D-1-deoxyxylulose are directly incorporated together into the C_5 skeleton of isoprenic units or whether D-1-deoxyxylulose is cleaved into two separate subunits before incorporation.

Enzymatic synthesis of ^{13}C labeled 1-deoxyxylulose

An enzyme activity producing D-1-deoxyxylulose was reported in numerous bacteria and fungi several years ago by Yokota and Sasajima.[5a-c] This enzyme of rather low specificity catalyzes the condensation of hydroxyethylthiamin obtained from pyruvate decarboxylation or from acetoin or methylacetoin cleavage on the carbonyl group of C_3, C_4 or C_5 aldoses (or the corresponding phosphates) of the D- as well as of the L-series. The *dxs* gene encoding the D-1-deoxyxylulose 5-phosphate synthase in *E. coli* was characterized and cloned as part of an operon that contains the *ispA* gene,[6] encoding the farnesyl diphosphate synthase, and two additional open reading frames encoding enzymes of unknown function (L.M. Lois, N. Campos, S. Rosa Putra, K. Danielsen, M. Rohmer and A. Boronat, unpublished results). The *dxs* gene has been cloned in an inducible expression vector and overexpressed in *E. coli*.[7a] As the enzymatic activity found in the induced strain was about 150-fold that of the wild-type strain, it was tempting to test a one-step enzymatic synthesis of ^{13}C labeled D-1-deoxyxylulose from labeled pyruvate and/or glyceraldehyde. This would be an elegant method to obtain doubly-labeled D-1-deoxyxylulose, using simultaneously ^{13}C labeled pyruvate and glyceraldehyde. Doubly-labeled [2,3-$^{13}C_2$]-D-1-deoxyxylulose has been previously obtained by a 14 steps chemical synthesis.[8]

A crude cell-free system obtained from the transformed *E. coli* strain was incubated in a buffer containing thiamin diphosphate,[7a] [2-^{13}C]pyruvate and either unlabeled D-glyceraldehyde, [1-^{13}C]- or [2-^{13}C]-DL-glyceraldehyde (that were the only available ^{13}C labeled glyceraldehyde forms), giving respectively [2-^{13}C]-, [2,3-$^{13}C_2$]- or [2,4-$^{13}C_2$]-D-1-deoxyxylulose in about 30% yield after TLC isolation.[9] Purification proved to be difficult. Pyruvate and glyceraldehyde could be removed, but unidentified minor products (representing about 20% of the sample according to ^{1}H and ^{13}C NMR) accompanied D-1-deoxyxylulose and were usually not removed as they did not interfere with the incorporation of the pentulose. D-1-Deoxyxylulose samples from enzymatic reaction were identified by ^{1}H and ^{13}C NMR spectroscopy of the free carbohydrate and GLC of the corresponding triacetate and comparison with data obtained on a sample of D-deoxyxylulose synthesized by the method of Broers and Arigoni[4] or found in the literature.[5,8] Both D- and L-glyceraldehyde were reported by Yokota and Sasajima to be respectively converted into D- and L-1-deoxyxylulose by a cell free system from *Bacillus pumilus* using DL-acetoin as an acyl donor.[5] With our system from *E. coli* using [2-^{13}C]pyruvate as an acyl donor, no detectable reaction occurred with L-glyceraldehyde as shown by TLC analysis (H_2SO_4/p-anisaldehyde detection) and ^{13}C-NMR, whereas D-1-deoxyxylulose was readily formed from D-glyceraldehyde and detected by the former methods. Our doubly labeled 1-deoxyxylulose samples were therefore assumed to possess mainly, if not completely, the D configuration.

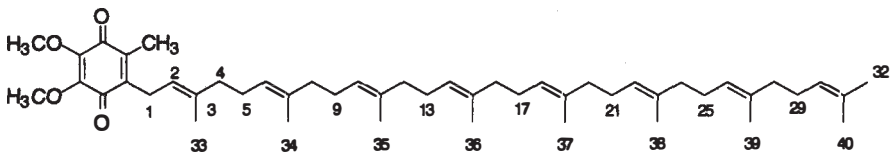


Figure 2. Ubiquinone Q8 from *Escherichia coli*.

Incorporations of [2-^{13}C]-, [2,3-$^{13}C_2$]- and [2,4-$^{13}C_2$]-D-1-deoxyxylulose into ubiquinone of *E. coli*

Addition of [2-^{13}C]-D-1-deoxyxylulose to the culture medium of a wild type strain of *E. coli* resulted in the labeling of all carbon atoms of ubiquinone Q8 (Figure 2) derived from C-3 of IPP, thus confirming its role as an isoprenoid precursor. The two doubly-labeled 1-deoxyxylulose samples were incubated separately, and ubiquinone was isolated from each culture.[10] In the ^{13}C-NMR spectrum of ubiquinone obtained from the culture grown in presence of [2,3-$^{13}C_2$]-D-1-deoxyxylulose, all carbon atoms derived from C-3 and C-4 of IPP were labeled. Their signals were doublets with a characteristic $^{13}C/^{13}C$ ^1J coupling constant (Figure 3). Due to the rather low incorporation rates (2-8%) of the labeled precursor in the presence of unlabeled glucose, these doublets corresponding to the enriched isoprenic units were accompanied by a singlet corresponding to the natural ^{13}C abundance and therefore to unlabeled isoprenic units. In presence of [2,4-$^{13}C_2$]-D-1-deoxyxylulose, carbon atoms derived from C-2 and C-3 of IPP were labeled, and again their signals corresponded to doublets with a characteristic ^1J coupling constant accompanied by singlet from unlabeled isoprenic units. Carbon atoms C-2, C-3 and C-4 of D-1-deoxyxylulose (corresponding to carbon atoms C-3, C-4 and C-2 of IPP) are thus incorporated together into isoprenic units. From previous labeling experiments with [4,5-$^{13}C_2$]- and [U-$^{13}C_6$]glucose, it was known that carbon atoms C-1, C-2 and C-4 from IPP on the one hand and C-3 and C-5 on the other hand are introduced together into the isoprenic skeleton. From all these data, one could deduce now that the five carbon atoms of the straight chain of D-1-deoxyxylulose correspond to those of IPP. Branching results from acyloin rearrangement. The $^{13}C/^{13}C$ coupling pattern observed after incorporation of the doubly labeled D-1-deoxyxylulose samples confirms the presence of such a transposition which is most likely the first committed step of the mevalonate-independent route, yielding probably 2*C*-methyl-D-erythritol 4-phosphate **4**, the first putative intermediate with the branched isoprenic skeleton.[11a,b] Indeed, the reaction catalyzed by the D-1-deoxyxylulose 5-phosphate synthase yields a compound that is not only involved in isoprenoid biosynthesis, but also in the formation of thiamine in bacteria and plants and pyridoxol in bacteria.[12a-c]

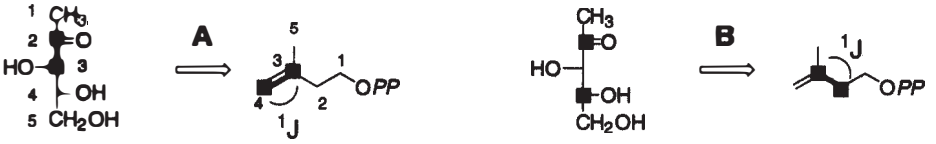


Figure 3. Labeling pattern of isoprenic units from ubiquinone Q8 (represented by the carbon skeleton of IPP) after incorporation of [2,3-$^{13}C_2$]-D-1-deoxyxylulose (A) or [2,4-$^{13}C_2$]-D-1-deoxyxylulose (B).

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7. (a) The coding region of the *E. coli* dxs gene was cloned into the expression vector pTACTAC.[7b] To create plasmid pTAC-DXS. *E. coli* XL1-Blue cells overexpressing the D-1-deoxyxylulose 5-phosphate synthase gene were grown for 20h under aerobic conditions at 37°C in a medium containing meat peptone (0.5%), meat extract (0.3%) and ampiciline (0.005%). Synthase expression was induced using isopropyl-β-D-thiogalactoside (0.2 mM) added after 6h of growth (0.5-0.8 optical density of the cultures). Cells were harvested by centrifugation, washed first with a 0.85% NaCl solution and than with buffer containing Tris-HCl (80 mM, pH=8), $MgCl_2$ (5 mM), EDTA disodium salt (2 mM) and phenylmethanesulfonyl fluoride (0.1 mM). Cells were lyophilized and stored at -30°C for up to 3 weeks without loss of synthase activity. Such lyophilized cells (0.6-0.8 g) were incubated with lysozyme (1 $mg.l^{-1}$) in the above mentioned buffer at 37°C for 30 min and disrupted by sonication at 4°C (40W, 1 min bursts followed by 2 min cooling in an ice bath). After centrifugation (18000 g, 4°C, 30 min) and addition of thiamin diphosphate (0.02 mmol), the resulting supernatant was incubated with sodium [2-^{13}C]pyruvate (162 mg, 1.8 mmol, ISOTEC Inc., Miamisburg, Ohio) and either unlabelled D-glyceraldehyde (198 mg, 1.8 mmol, Fluka), [1-^{13}C]-DL-glyceraldehyde (1.8 mmol, ISOTEC Inc.) or [2-^{13}C]-DL-glyceraldehyde (1.8 mmol, Omicron Biochemicals Inc. South Bend, Indiana) with constant stirring at 37°C for 20 h. Isotopic abundance of all starting products was 99%. Reaction was quenched by heating for 5 min at 80°C. Denatured proteins were removed by centrifugation (18000 g, 30 min), and the supernatant was lyophilized. (b) Browner, M.F.; Rasor, P.; Tugendreich, S.; Fletterick, R.J., *Protein Engin.*, **1991**, *4*, 351-357.
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9. Isolation and characterization of ^{13}C labeled D-1-deoxyxylulose samples. The crude lyophilized reaction mixtures were extracted with methanol (3x25 ml), The solvent was removed under reduced pressure, and D-1-deoxyxylulose isolated by preparative silica-gel TLC on hand-made plates (Merck PF254, 0.5 mm thickness, $CHCl_3/CH_3OH$, 8:2, R_f=0.40) yielding [2-^{13}C]-D-1-deoxyxylulose (77 mg), [2,3-$^{13}C_2$]-D-1-deoxyxylulose (85 mg) or [2,4-$^{13}C_2$]-D-1-deoxyxylulose (73 mg). All experiments were repeated twice in order to get enough material for the incubation experiments. Only the open form, representing about 80% of the material,[6] is described in the NMR spectra. The two hemiketals were observed as minor constituents. For the ^{1}H-NMR spectra, only the well resolved signals from the methyl group were indicated.
[2-^{13}C]-D-1-Deoxyxylulose. ^{1}H NMR (200 MHz, D_2O): δ = 2.08 (d, $^2J(^1H/^{13}C)$= 6Hz, CH_3-^{13}CO-) ; ^{13}C NMR (50 MHz, D_2O): δ = 212.2 (s, C-2). [2,3-$^{13}C_2$]-D-1-Deoxyxylulose. ^{1}H NMR (200 MHz, D_2O): δ = 2.05 (dd, $^2J(^1H/^{13}C)$=6Hz, $^3J(^1H/^{13}C)$=1Hz, CH_3-^{13}CO-^{13}CHOH-); ^{13}C NMR (50 MHz, D_2O): δ = 212.2 (d, J=40.6 Hz, C-2), 76.3 (d, J=40.7 Hz, C-3). [2,4-$^{13}C_2$]-D-1-Deoxyxylulose. ^{1}H NMR (200 MHz, D_2O): δ = 2.05 (d, $^2J(^1H/^{13}C)$= 6Hz, CH_3-^{13}CO-); ^{13}C NMR (50 MHz, D_2O): δ = 212.0 (s, C-2), 70.4 (s, C-4) (no ^2J $^{13}C/^{13}C$ coupling was observed).
10. Ubiquinone labeling experiments with ^{13}C labeled D-1-deoxyxylulose. *E. coli* (strain DSM 30083) was grown at 37°C for 24 h in a medium containing Na_2HPO_4 (1.7%), KH_2PO_4 (0.3%), NaCl (0.05%), NH_4Cl (0.2%), $MgSO_4.7H_2O$ (0.0049%), $CaCl_2.2H_2O$ (0.0015%), D-glucose (3 g) and either [2-^{13}C]-D-1-deoxyxylulose (150 mg), [2,3-$^{13}C_2$]-D-1-deoxyxylulose (165 mg) or [2,4-$^{13}C_2$]-D-1-deoxyxylulose (150 mg). Ubiquinone 8 (1.4-1.7 mg) was obtained from lyophilized cells (200 mg, dry weight) from 1l cultures supplemented with either [2-^{13}C]-, [2,3-$^{13}C_2$]- or [2,4-$^{13}C_2$]-D-1-deoxyxylulose samples as previously described.[1b]
In the following ^{13}C NMR spectra of labeled ubiquinone, signals bearing a superscript * were utilized as internal reference for the evaluation of isotopic abundances.[1a-c] Those that are not mentioned were to weak to be observed. The ubiquinone spectra obtained after the two incorporations of doubly labeled D-1-deoxyxylulose resulted from the superposition of the signals of unlabeled isoprenic units (corresponding to the singlets) with those from labeled isoprenic units in which the signals of the labeled carbon atoms were doublets.
^{3}C NMR of ubiquinone (100MHz, $CDCl_3$)
Incorporation of [2-^{13}C]-D-1-deoxyxylulose. δ = 137.68 (C-3, 6%), 135.29-134.97 (C-7,11,15,19,23 and 27, 3%), 131.28 (C-31, 3%), 124.30 (C-10,14,18,22,26 and 30, 1%), 61.14* (2x-OCH_3, 1.1%), 39.76 (C-4,8,12,16,20,24 and 28, 1%), 26.74 (C-5,9,13,17,21,25 and 29, 1%), 16.04 (C-34,35,36,37,38 and 39, 1%).
Incorporation of [2,3-$^{13}C_2$]-D-1-deoxyxylulose. δ = 134.95 (C-7,11,15,19,23, and 27, d, J=45.0Hz, 2%) + 134.98 (s, 1.1%), 124.30 (C-10,14,18,22,26 and 30, s, 1.7%), 118.9 (C-2, s, 2.7%), 61.16 (2x-OCH3, s, 2%), 39.74 (C-4,8,12,16,20,24 and 28, d, J=43.4Hz, 2.3%] + 39.75 (s, 1.0%), 26.74 (C-5,9,13,17,21,25 and 29, 1.3%), 25.72 (C-32, d, J=43.4Hz, 3.0%) + 25.71 (s, 1.1%), 16.04* (C-34,35,36,37,38 and 39, s, 1.1%).
Incorporation of [2,4-$^{13}C_2$]-D-1-deoxyxylulose. δ = 137.65 (C-3, d, J=73.9Hz, 11%) + 137.68 (s, 6%), 134.95 (C-7,11,15,17,23 and 27, d, J=72.3Hz, 7%) + 134.97 (s, 1%), 131.26 (C-31, d, J=73.9Hz, 9%), 124.27 (C-10,14,18,22,26 and 30, d, J=73.9Hz, 9%) + 124.30 (s, 3%), 118.86 (C-2, d, J=73.9Hz, 15%) + 118.89 (s, 4%), 39.75* (C-4,8,12,16,20,24 and 28, s, 1.1%), 26.74 (C-5,9,13,17,21,25 and 29, s, 1.2%), 16.04 (C-34,35,36,37,38 and 39, s, 0.9%).
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