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Incorporation of 2-*C*-Methyl-D-erythritol, a Putative Isoprenoid Precursor in the Mevalonate-Independent Pathway, into Ubiquinone and Menaquinone of *Escherichia coli*

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Abstract: Incorporation of deuterium labelled 2-*C*-methyl-D-erythritol into isoprenoid side-chains of ubiquinone and menaquinone from *Escherichia coli* strongly supports the proposed intermediate role of this branched sugar derivative in the mevalonate independent pathway for isoprenoid biosynthesis via glyceraldehyde 3-phosphate and pyruvate. © 1997 Published by Elsevier Science Ltd.

It is generally accepted that isopentenyl diphosphate (IPP), the universal precursor of isoprenoids, derives from acetyl-CoA and mevalonate. The discovery of a mevalonate-independent route in bacteria was therefore a great surprise.[1a] The operation of this route was also later found in green algae[1c] and in higher plants, but only for the biosynthesis of chloroplast-linked isoprenoids[1d], diterpenoids[1e,f] and monoterpenoids.[1g] The role of 1-deoxyxylulose or its 5-phosphate, resulting from a condensation of hydroxethyl thiamine on glyceraldehyde 3-phosphate (GAP),[1b] as a C_5 precursor for IPP was shown by the successful incorporation of deuterium-labelled 1-deoxyxylulose into the isoprenoids of *Escherichia coli*,[2] and more recently of the mixture of deuterium-labelled methyl α- and β-1-deoxy-D-xylulosides into isoprene emitted by leaves of higher plants.[3] However, the remaining steps between 1-deoxyxylulose 5-phosphate and IPP remained obscure. 2-*C*-Methyl-D-erythritol derivatives have been detected in several bacteria and plants under normal growth conditions as well as in response towards stress.[4,5] This branched polyol fits perfectly into our biogenetic scheme: 2-*C*-methylerythritol 4-phosphate could derive from 1-deoxyxylulose 5-phosphate by a rearrangement followed by reduction, much like the reactions involved in the formation of the carbon skeleton of valine (Fig. 1).[1a,b] Our recent results on the biosynthesis of 2-*C*-methylerythritol support this hypothesis.[6] Feeding experiments with *Corynebacterium ammoniagenes* using [1-^{13}C]-, [6-^{13}C]- and [U-$^{13}C_6$]glucoses revealed identical labelling patterns in the isoprenoid side-chains of dihydromenaquinones and the carbon skeleton of 2-*C*-methylerythritol, in full accordance with the GAP/pyruvate pathway.

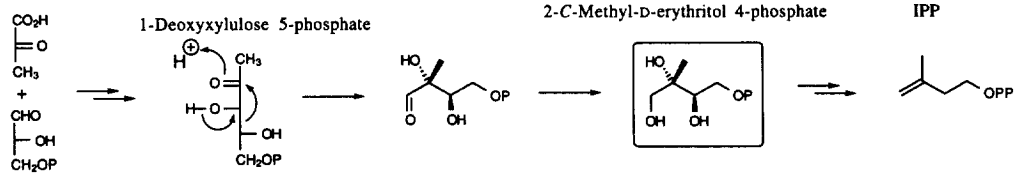

Fig. 1. Hypothetical biogenetic scheme for the GAP/pyruvate pathway.

In order to confirm this proposed intermediate role, incorporation of deuterium-labelled racemic, D- or L- 2-*C*-methylerythritol into isoprenoids of bacteria possessing the mevalonate independent route were attempted. No incorporation occurred into the isoprenoids of *C. ammoniagenes* or *Methylobacterium organophilum*, but positive results were obtained with *E. coli*. The first incorporation experiments were carried out using racemic [1,1-$^2H_2$]methyl-DL-erythritol which was synthesized by using a related strategy to those described by Anthonsen *et al.*[7] OsO_4 mediated dihydroxylation of 3-methylfuran-2(5*H*)-one **1** followed by $LiAlD_4$

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reduction yielded [1,1-$^2H_2$]methyl-DL-erythritol. Cultures of *E. coli* (DSM 30083), grown on a synthetic medium as previously described[1a] with glucose as sole carbon source (1 g/l), were supplemented with [1,1-$^2H_2$]-2-*C*-methyl-DL-erythritol (600 mg/l). Ubiquinone-8 **6** (Fig. 3) was isolated according to established procedures.[1a] The mass spectrum of ubiquinone showed deuterium incorporation monitored by the increased relative intensities of ions corresponding to M+2 and M+4 of ubiquinone in comparison with non-labelled compound. Also the ^2H NMR spectrum of ubiquinone gave evidence for deuterium labelling on carbon atoms corresponding to C-4 of IPP, in accordance with the proposed biogenetic scheme.

Motivated by these preliminary results, we decided to synthesize both methyl-D- and L-erythritol for separate feeding experiments (Fig. 2).[8] 3-Methylfuran-2(5*H*)-one **1** was reduced with $LiAlD_4$ at 0°C yielding the desired unsaturated diol in 85% yields accompanied with the saturated diol in only 6-8% yields. Sharpless enantioselective dihydroxylation of the corresponding diacetate **3** using commercial AD-mix reagents[9] afforded 2-*C*-methylerythritol diacetate **4a** or **4b** with satisfactory enantiomeric excess (80% e.e.), much better than those obtained with the diol (31% e.e.) or the dibenzoate (60% e.e.), and in almost quantitative chemical yields. Partial intramolecular transesterification of the acetate groups in the dihydroxylation product **4a** or **4b** led to a mixture of diacetates which were isolated together and quantitatively deacetylated with basic ion exchange resin yielding [1,1-$^2H_2$]-2-*C*-methyl-D-erythritol (from AD-mix-ß) or [1,1-$^2H_2$]-2-*C*-methyl-L-erythritol (from AD-mix-α). Enantiomeric excesses of 2-*C*-methylerythritol samples were monitored by ^1H NMR spectroscopy of the triacetates supplemented with Eu(hfc)$_3$ chiral shift reagent (1 eq.). The complexes with the tertiary hydroxy group were characterised by different chemical shifts for the C-2 methyl groups from the two enantiomers. ^1H NMR and GC analysis of the diastereomeric esters obtained by esterification of the secondary hydroxy group in both enantiomers of the major 2-*C*-methylerythritol diacetates **4a** or **4b** with (*R*) or (*S*)-α-methoxy-α-trifluoromethylphenyl acetyl chloride[10] confirmed the above mentioned 80% e.e. In order to compare the absolute configuration of synthetic 2-*C*-methylerythritols and the naturally occurring compound with the supposed D-erythro configuration isolated from *C. ammoniagenes*,[6] Eu(hfc)$_3$ and minor quantities of the synthetically prepared compound with the supposed L-erythro configuration were added to the triacetate of the natural compound. The synthetic compound with the supposed L-erythro configuration gave rise to an additional methyl peak in the ^1H NMR spectrum with upfield chemical shift compared with the methyl group of the natural compound supporting the supposed D-erythro configuration of the natural compound. Optical rotation measurements of the free tetrol revealed a positive sign for the supposed D-erythro isomer and a negative sign for the supposed L-erythro isomer, in accordance with literature reports.[5b,11]

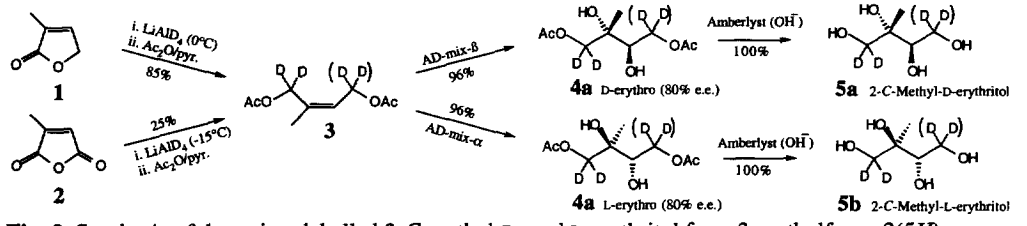

Fig. 2. Synthesis of deuterium labelled 2-*C*-methyl-D- and L-erythritol from 3-methylfuran-2(5*H*)-one or citraconic anhydride. Deuterium atoms in parentheses originated from the reduction of citraconic anhydride.

Because of the unavoidable reduction of ubiquinone into ubiquinol by recording mass spectra,[12] [$^2H_4$]-2-*C*-methylerythritol should give more accurate results for mass spectrometric analysis. 2-*C*-Methylerythritol simultaneously labelled with two deuterium atoms at both carbons C-1 and C-4 was therefore synthesized. Reduction of dimethylcitraconate with $LiAlD_4$ afforded low yields of a mixture of diols in which the saturated diol was the major compound. Direct dihydroxylation of dimethylcitraconate was unsuccessful with the AD-mix reagents employed. Citraconic anhydride **2** (fig. 2) which is somehow more easily reduced, afforded predominantly the unsaturated diol upon reduction with $LiAlD_4$ at -15°C. Enantioselective dihydroxylation of the diacetate **3** followed by deacetylation was carried out as usual to yield [1,1,4,4-$^2H_4$]-2-*C*-methyl-D-erythritol **5a** or [1,1,4,4-$^2H_4$]-2-*C*-methyl-L-erythritol **5b**. Low yields in the reduction step limited the value of this strategy on a multigram scale.

The D- and L-enantiomers, each labelled with four deuterium atoms, were supplemented (300mg/l) to separate cultures of *E. coli* (200 ml). A reference culture was grown in absence of 2-*C*-methylerythritol but otherwise identical growth conditions. Ubiquinone samples from the three cultures were isolated and analysed for their deuterium labelling by means of fast atom bombardment mass spectroscopy (FAB MS).[13] This milder ionisation method induced less fragmentations and therefore allowed more accurate detection of eventual additional molecular ions resulting from deuterium labelling. The FAB mass spectrum of the non-labelled reference revealed furthermore high degree of reduction of ubiquinone to ubiquinol (ca. 80%). More reliable data were therefore obtained by analysing the molecular ion of ubiquinol and its additional heavier ions resulting from labelling. The mass spectrum of ubiquinone from the culture supplemented with the D-erythro isomer revealed the presence of additional ions corresponding to M+4 and M+8 of ubiquinol with low, but significant relative intensities (incorporation rate ca. 10%). These heavier molecular ions were consistent with the incorporation of one and two molecules of intact 2-*C*-methylerythritol per labelled ubiquinone with retention of all four deuterium atoms. No measurable labelling of ubiquinone occured in the culture supplemented with the L-enantiomer.

In order to complete the results obtained by FAB MS, a large scale culture (6 l) of *E. coli* was supplemented with [1,1-$^2H_2$]-2-*C*-methyl-D-erythritol (300 mg/l). Sufficient quantities of ubiquinone (1.6 mg) and menaquinone **7** (0.7 mg) were obtained for ^2H NMR analysis.[14] The ^2H NMR spectra of both quinones clearly confirmed deuterium labelling only at the carbon atoms corresponding to C-4 of IPP (Fig. 3).

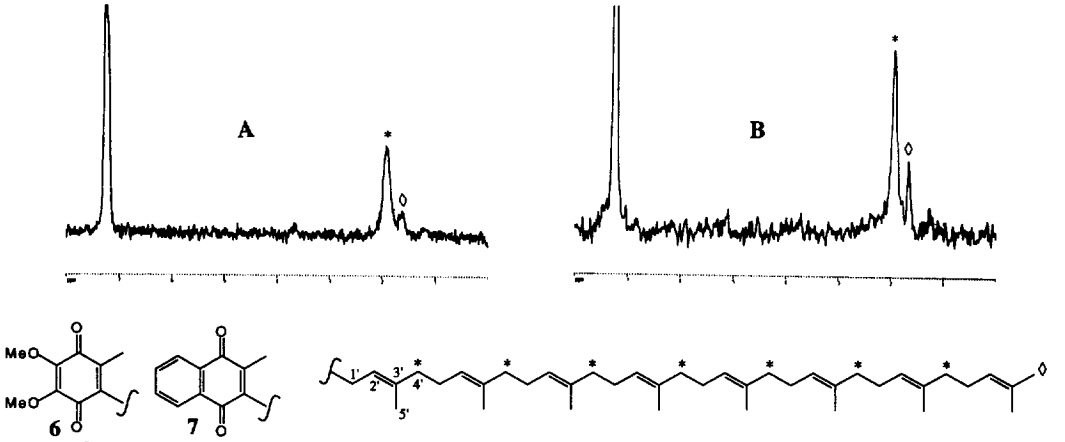

Fig. 3. ^2H NMR (77 MHz, $CHCl_3$) **A** of ubiquinone **6** and **B** of menaquinone **7** labelled from [1,1-$^2H_2$]-2-*C*-methyl-D-erythritol. The locations of deuterium atoms in the isoprenoid side-chain are shown by * and ◊.

The successful incorporation of 2-*C*-methyl-D-erythritol into the isoprenoid side-chains of ubiquinone and menaquinone from *E. coli* shows that the carbon skeleton of IPP is directly derived from 2-*C*-methylerythritol or a 2-*C*-methylerythritol derivative. These results are in accordance with our recent results from feeding experiments with *C. ammoniagenes* which clearly revealed the formation of 2-*C*-methyl-D-erythritol and IPP via the GAP/pyruvate pathway. It cannot be excluded that 2-*C*-methylerythritol is not itself the precursor of IPP, but can be readily converted into an IPP precursor. Indeed, the rearragement step (Fig. 1) occurs most likely on 1-deoxyxylulose 5-phosphate, yielding 2-*C*-methylerythritol 4-phosphate. No incorporation into the isoprenoids of *M. organophilum* and *C. ammoniagenes*, and a rather modest incorporation into those of *E. coli*, could be due to the absence of an efficient kinase required for the formation of 2-*C*-methylerythritol 4-phosphate, the actual precursor.

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References and Notes

(1) (a) Rohmer, M.; Knani, M.; Simonin, P.; Sutter, B.; Sahm, H., *Biochem. J.*, **1993**, *295*, 517-524. (b) Rohmer, M.; Seemann, M.; Horbach, S.; Bringer-Meyer, S.; Sahm, H., *J. Am. Chem. Soc.* **1996**, *118*, 2564-2566. (c) Schwender, J.; Seemann, M.; Lichtenthaler, H.K.; Rohmer, M., *Biochem. J.*, **1996**, *316*, 73-80. (d) Lichtenthaler, H.K.; Schwender, J.; Disch, A.; Rohmer, M., *FEBS Letters*, **1997**, *400*, 271-274. (e) Schwarz, M.K., Ph.D. Thesis, Eidgenössische Technische Hochschule, Zürich, Switzerland, **1994**. (f) Eisenreich, W.; Menhard, B.; Hylands, P.J.; Zenk, M.H.; Bacher, A., *Proc. Natl. Acad. Sci. USA*, **1996**, *93*, 6431-6436. (g) Eisenreich, W.; Sagner, S.; Zenk, M.H.; Bacher, A., *Tetrahedron Lett*, **1997**, *38*, 3889-3892.

(2) Broers, S.T.J., Ph.D. Thesis, Eidgenössische Technische Hochschule, Zürich, Switzerland, **1994**.

(3) Zeidler, J.G.; Lichtenthaler, H.K.; May, H.U.; Lichtenthaler, F.W., *Z. Naturforsch.*, **1997**, *52c*, 15-23.

(4) (a) Ostrovsky, D.; Kharatian, E.; Malarova, I.; Shipanova, I.; Sibeldina, L.; Shashkov, A.; Tantsirev, G., *BioFactors*, **1992**, *3*, 261-264. (b) Turner, L.L.; Santos, H.; Fareleira, P.; Pacheco, I.; Le Gall, J.; Xavier, A.V., *Biochem. J.*, **1992**, *285*, 387-390.

(5) (a) Ford, C., *Phytochemistry*, **1981**, *20*, 2019-2020. (b) Dittrich, P.; Angyal, S.J., *Phytochemistry*, **1988**, *27*, 935. (c) Kitajima, J.; Tanaka, Y., *Chem. Pharm. Bull.*, **1993**, *41*, 1667-1669.

(6) Duvold, T.; Bravo, J.-M.; Pale-Grosdemange, C.; Rohmer, M., *Tetrahedron Lett.*, in press.

(7) (a) Anthonsen, T.; Hagen, S.; Kazi, M.A.; Shah, S.W.; Tagar, S., *Acta Chem. Scand.*, **1976**, *B30*, 91-92. (b) Anthonsen, T.; Hagen, S.; Sallam, M.A.E., *Phytochemistry*, **1980**, *19*, 2375-2377.

(8) **Preparation of diacetate 3, general procedure.** 3-Methylfuran-2(5*H*)-one **1** and citraconic anhydride **2** were reduced with an excess of $LiAlH_4$ or $LiAlD_4$ in dry ether at 0°C (furanone) or at -15°C (citraconic anhydride) until all starting material was consumed (2-5 h). The reaction was then quenched by slow addition of a saturated aq. solution of Na_2SO_4 until the appearance of a white granular precipitate and stirring was continued for 30 min. The precipitate was filtered off, the filtrate was dried (Na_2SO_4) and ether was evaporated under reduced pressure yielding a colourless oil of crude diol which was directly acetylated (pyr/Ac_2O, 1:1, v/v). Purification by flash column chromatography (FCC) (hexane/EtOAc, 4:1) afforded an inseparable mixture of diacetate **3** (85% yields from furanone, 25% yields from citraconic anhydride) and the corresponding saturated diacetate (7% yields from furanone, 5% yields from citraconic anhydride) (the ratio saturated/unsaturated diols were judged from the ^1H NMR spectra).^1H NMR (200MHz, $CDCl_3$): δ = 1.81 (3H, d, $J_{5\text{-}3}$=0.9 Hz, 5-H), 2.04 and 2.05 (6H, 2s, -$COCH_3$, x2), 4.62 (2H, s, H-1), 4.63 (2H, d, $J_{4\text{-}3}$=7.0 Hz, H-4), 5.55 (1H, dt, $J_{3\text{-}4}$=7.0 Hz and $J_{3\text{-}5}$=0.9 Hz, H-3); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 20.97 (C-5), 21.45 (-OCO<u>C</u>H_3, x2), 60.32 (C-4), 62.68 (C-1), 123.95 (C-3), 136.68 (C-2), 170.83 (-O<u>C</u>OCH_3, x2).

Dihydroxylation of diacetate 3 by the general procedure of Sharpless.[9] Enantiomeric dihydroxylations were conducted at 0°C in a mixture of t-BuOH/H_2O (1:1, v/v) using commercial AD-mix-α or AD-mix-ß reagents (Aldrich) and $CH_3SO_2NH_2$. The reaction was completed within 40 h stirring at 0°C, and the reaction was after this time quenched by the addition of solid Na_2SO_3. Repeated extractions with EtOAc, drying of the combined extracts (Na_2SO_4) and evaporation of solvents under reduced pressure afforded a crude yellow oil which crystallised upon standing. Purification by FCC (EtOAc/hexane, 9:1) afforded a mixture of diacetates due to partial intramolecular transesterification in **4a** and **4b** (96% yields). The combined diacetates were deacetylated overnight with activated Amberlyst A-26 (OH^-) in MeOH affording pure methylerythritol as a colourless oil which crystallised upon standing (100% yields). 2-*C*-Methylerythritol triacetate was obtained by acetylation of the tetrol for 2 h at rt. (pyr./Ac_2O, 1:1, v/v). Triacetate: ^1H NMR (200 MHz, $CDCl_3$) δ = 1.23 (3H, s, 5-H), 2.03, 2.08 and 2.10 (9H, s, -$COCH_3$, x3), 2.57 (1H, s, -OH), 3.89 (1H, d, $J_{1a\text{-}1b}$=11.6 Hz, 1-H_a), 4.14 (1H, d, $J_{1b\text{-}1a}$=11.6 Hz, 1-H_b), 4.15 (1H, dd, $J_{4a\text{-}4b}$=12.0 Hz, $J_{4a\text{-}3}$=8.0 Hz, 4-H_a), 4.56 (1H, dd, $J_{4b\text{-}4a}$=12.0 Hz, $J_{4b\text{-}3}$=2.7 Hz, 4-H_b), 5.17 (1H, dd, $J_{3\text{-}4a}$=8.0 Hz, $J_{3\text{-}4b}$=2.7 Hz, 3-H). The following ^{13}C NMR assignments were supported by supplementary experiments including DEPT, HMQC, HMBC of the corresponding tetraacetate (obtained by acetylation overnight at 60°C) and comparison with the ^{13}C NMR spectrum of the natural compound.[6] ^{13}C NMR (50 MHz, $CDCl_3$) δ = 20.04 (5-C), 20.82 (-OCO<u>C</u>H_3, x3), 62.77 (4-C), 66.70 (1-C), 72.24 (2-C), 72.73 (3-C), 170.06, 170.86 and 171. 97 (-O<u>C</u>OCH_3, x3). Also the free tetrols showed satisfactory ^1H and ^{13}C NMR spectra. The locations of deuterium atoms in the corresponding labelled compounds were determined by the absence of proton signals from the labelled sites and the modified coupling patterns of neighbouring protons in the ^1H NMR spectra. Upfield chemical shifts for carbon atoms placed ß to the deuterium atoms (ß-shift) and absence of carbon signals for deuterium bearing carbon atoms (due to signal ^{13}C-^2H multiplicity and longer relaxation time) in the ^{13}C NMR spectra confirmed the location of deuterium atoms. 2-*C*-Methyl-D-erythritol: $[\alpha]_D^{20}$ = +7.0 (c=1.6, MeOH), Lit.[5b] +14.6 (c=1.37, MeOH), 2-*C*-methyl-L-erythritol: $[\alpha]_D^{25}$ = -6.8 (c=1.2, MeOH).

(9) Kolb, H.C.; VanNieuwenhze, M.S.; Sharpless, K.B., *Chem. Rev.*, **1994**, *94*, 2483-2547, and references cited.

(10) Dale, J.L.; Dull, D.L.; Mosher, H.S., *J. Org. Chem.*, **1969**, *34*, 2543-2549.

(11) (a) Shah, S.W.; Brandänge, S.; Behr, D., Dahmén, J.; Hagen, S.; Anthonsen, T., *Acta Chem. Scand.*, **1976**, *B30*, 903. (b) Anthonsen, T.; Hagen, S.; Lwande, W., *Acta. Chem. Scand.*, **1980**, *B34*, 41-45. (c) Ostrovsky, D.; Shashkov, A.; Sviridov, A., *BJ Letters*, **1993**, 901-902.

(12) Muraca, R.F.; Whittick, J.S.; Doyle Daves, Jr., G.; Friis, P.; Folkers, K., *J. Am. Chem. Soc.*, **1967**, *89*, 1505-1508.

(13) Ubiquinone was analysed by positive FAB ion mass spectrometry on a ZAB-HF spectrometer (m-nitrobenzyl alcohol matrix, xenon, 8 keV). Compound labelled from [1,1,4,4-$^2H_4$]-2-*C*-methyl-D-erythritol: *m/z* = 728.3 (M of ubiquinol, 100%), 729.3 (M+1, 54%), 730.3 (M+2, 15%), 732.3 (M+4, 11%), 734.3 (M+8, 3%). Unlabelled compound: *m/z* = 728.3 (M of ubiquinol, 100%), 729.3 (M+1, 54%), 730.3 (M+2, 15%). Calculated incorporation rate was 10% based on the increased relative intensities of M+4 and M+8 of ubiquinol from the labelled ubiquinone by comparison with the spectrum of the non-labelled compound.

(14) Ubiquinone (R_f 0.61) and menaquinone (R_f 0.88) were purified by TLC (CH_2Cl_2, 100%). EI MS (15 eV) of menaquinone labelled with [1,1-$^2H_2$]-2-*C*-methyl-D-erythritol: *m/z* = 715.7 (M, 100%), 716.6 (M+1, 60%), 717.6 (M+2, 28%). Unlabelled compound *m/z* = 715.7 (M, 100%), 716.6 (M+1, 57%), 717.6 (M+2, 19%).

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