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Biosynthesis of 2-*C*-Methyl-D-erythritol, a Putative C_5 Intermediate in the Mevalonate Independent Pathway for Isoprenoid Biosynthesis

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Abstract: According to incorporations of ^{13}C labelled glucose, 2-*C*-methyl-D-erythritol was most likely synthesized by *Corynebacterium ammoniagenes* from glyceraldehyde 3-phosphate and from pyruvate via a condensation and a rearrangement. The observed labelling patterns for methylerythritol were identical with those of the isoprenic units of dihydromenaquinones from the same bacterium, suggesting for this tetrol an intermediate role in the non-mevalonate pathway for isoprenoid biosynthesis. © 1997 Published by Elsevier Science Ltd.

Isoprenoids are widespread in all living organisms, often as essential metabolites.[1] Isopentenyl diphosphate (IPP), the common precursor of all isoprenoids, was generally believed to arise from acetyl coenzyme A, 3-hydroxy-3-methylglutaryl-coenzyme A, and mevalonate.[2] Recent studies, however, revealed that this generally accepted pathway was not operating in all living organisms, and that isoprenoids could be formed via a mevalonate independent pathway in most investigated bacteria, in green algae and chloroplasts of higher plants.[3] Glyceraldehyde 3-phosphate (GAP) and pyruvate were recently identified as precursors for IPP in this metabolic route, yielding after condensation of (hydroxyethyl)thiamine on GAP, 1-deoxyxylulose 5-phosphate.[3f] Cell free systems from many bacteria, including *Escherichia coli*, fungi and yeasts were known to produce 1-deoxyxylulose from D-glyceraldehyde and pyruvate.[4] In addition, Broers and Arigoni have shown that deuterium labelled 1-deoxyxylulose was incorporated into the prenyl side-chain of ubiquinone and menaquinone from *E. coli* with a labelling in accordance with the non-mevalonate pathway.[5] 1-Deoxyxylulose 5-phosphate derived from a condensation of GAP and pyruvate is therefore most probably the first C_5 intermediate in this metabolic route.

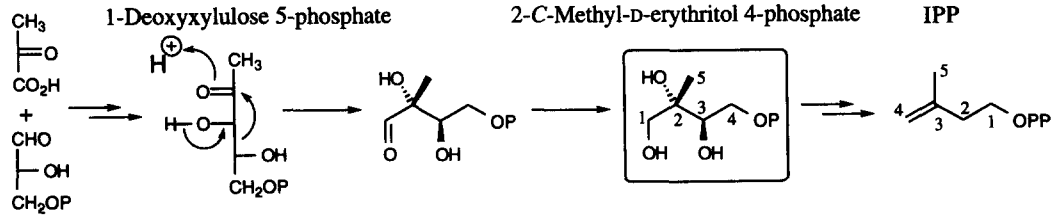

Figure 1. Hypothetical biogenetic scheme for the formation of 2-*C*-methyl-D-erythritol and IPP in the mevalonate independent pathway for isoprenoid biosynthesis.

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A cyclic diphosphate of 2-*C*-methyl-D-erythritol has been detected in *Desulfovibrio desulfuricans* in normal growth conditions as well as in several bacteria in very high concentrations in response to an oxidative stress induced by benzylviologen.[6] The free tetrol or the corresponding lactone were also found in higher plants, quite often in stress conditions (senescent leaves, water stress).[7] Nothing is known about this compound, neither on its biological role, nor on its biosynthesis.[6-8] Methylerythritol fits however perfectly into the biogenetic scheme which we recently proposed for the isoprenoid biosynthesis via the GAP/pyruvate pathway. According to this scheme, methylerythritol 4-phosphate should derive directly from 1-deoxyxylulose 5-phosphate upon a transposition reaction followed by a reduction (Fig. 1).

Corynebacterium ammoniagenes, which is able to accumulate methylerythritol-2,4-cyclodiphosphate upon treatment with benzylviologen during the stationary growth phase,[6] seemed to be an ideal model for labelling experiments since it also produces dihydromenaquinone-8, MK-8 (II-H_2), and dihydromenaquinone-9, MK-9 (II-H_2).[9] This allowed direct comparison between the labelling patterns of the methylerythritol skeleton with those of the isoprenic units of MK-8/9 (II-H_2). 2-*C*-Methylerythritol tetraacetate and the dihydromenaquinones were isolated from *C. ammoniagenes* (DSM 20305) supplemented with benzylviologen hydrochloride (50 mg/l) during the stationary phase.[6b,11] Feeding experiments using [1-^{13}C]-, [6-^{13}C]- and [U-$^{13}C_6$]glucose were carried out, and the labelling patterns of the isolated compounds were analysed by means of ^{13}C NMR spectroscopy and ^{1}H/^{13}C heteronuclear multiple bond coherence (HMBC) NMR spectroscopy.

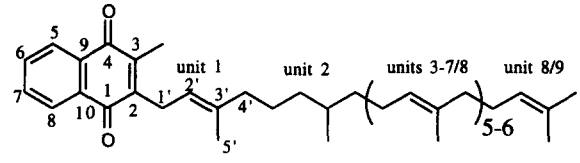

Figure 2. Dihydromenaquinones from *Corynebacterium ammoniagenes*.

Incorporations of [1-^{13}C]- or [6-^{13}C]glucose (isotopic abundance 15%) led to the same labelling patterns in 2-*C*-methylerythritol tetraacetate and in the isoprenic units of MK-8/9 (II-H_2) (Fig. 2). Both series of compounds were labelled on corresponding carbon atoms: C-4 and C-5 of methylerythritol, carbon atoms derived from C-1 and C-5 of IPP for the dihydromenaquinones. These data were in accordance with 1) glucose main catabolism via glycolysis, as expected for Corynebacteria, with some contribution of the oxidative pentose phosphate pathway which accounts for the more efficient incorporation of C-6 than C-1 of glucose that is lost by decarboxylation in the non-oxidative pentose phosphate pathway, 2) the formation of the branched C_5 skeleton of methylerythritol and of the dihydromenaquinone isoprenic units via the GAP/pyruvate pathway and 3) finally the absence of the acetate/mevalonate route for isoprenoid biosynthesis. Similar incubation experiments afforded similar labelling patterns in the isoprenoids from *E. coli* and *Alicyclobacillus acidoterrestris* which utilise glucose by the same metabolic routes and were shown to synthesise their isoprenoids by the non-mevalonate pathway.[3d]

Evidence for the formation of methylerythritol from two distinct subunits (C_2 and C_3) was obtained by incorporation of [U-$^{13}C_6$]glucose (99% isotopic abundance, diluted in a 18:85 ratio with unlabelled glucose). The ^{13}C NMR spectrum of 2-*C*-methylerythritol tetraacetate labelled from [U-$^{13}C_6$]glucose (Fig. 3) revealed one bond coupling between C-2 and C-5 ($^{1}J_{C\text{-}C}$=38.2 Hz) as well as C-3 and C-4 ($^{1}J_{C\text{-}C}$=44 Hz). Long range coupling was also observed between C-1 and C-4 ($^{3}J_{C\text{-}C}$=1.8 Hz) and proved the simultaneous insertion of C-1 and C-4, and most probably also C-3, from a single C_3 precursor. Expected long range coupling between C-1 and C-3 ($^{2}J_{C\text{-}C}$), however, could not be observed. This problem was overcome by employing an indirect method recently described by Seto *et al.*[12] HMBC modified by removing the low-pass J-filter can be used to observe direct ^{13}C-^{1}H long-range couplings. The modified HMBC spectrum of 2-*C*-methylerythritol tetraacetate revealed

a cross peak due long range coupling between C-1 and H-3 accompanied with additional satellite peaks ($^1J^{13}_{C\text{-}^1H}$ = 140 Hz). The cross peak represented the long distance coupling between C-1 and H-3 when H-3 is bound to a ^{12}C while the satellite peaks are due to the long range coupling between C-1 and H-3 when H-3 is attached to a ^{13}C carbon atom. This gives additional proof that C-1, C-3 and C-4 were simultaneously incorporated and therefore originated from the same C_3 precursor.

The ^{13}C NMR spectrum of MK-8/9 (II-H_2) labelled from [U-$^{13}C_6$]glucose (Fig. 3) revealed long range couplings in the isoprenic units (calculated from unit 1,2 and terminal) between the carbon atoms derived from C-1 and C-4 of IPP ($^3J_{C\text{-}C}$) and C-2 and C-4 ($^2J_{C\text{-}C}$) in addition to one bond couplings between those derived from C-1 and C-2 or C-3 and C-5. This proves unambiguously the operation of the GAP/pyruvate pathway for the formation of the isoprenic units of MK-8/9 (II-H_2). Cross peaks between H-2 and C-4 in isoprenic unit 1 and in the terminal unit were observed with additional satellite peaks in the modified HMBC spectrum of MK-8/9 (II-H_2) (unit 1: $^1J_{^{13}C\text{-}^1H}$ = 160 Hz, terminal unit: $^1J_{^{13}C\text{-}^1H}$ = 166 Hz), thus giving additional proofs for the origin of C-2 and C-4 of IPP from the same glucose molecule and hence for the operation of the GAP/pyruvate pathway.

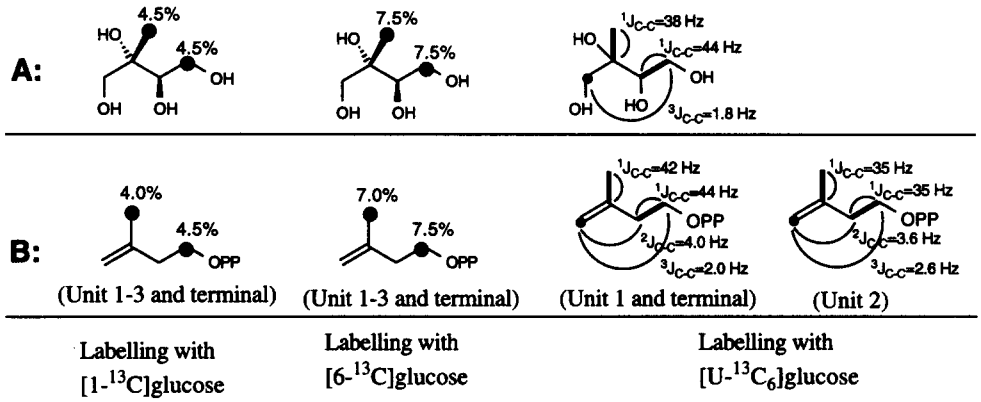

Figure 3. Isotopic enrichments and $^{13}C/^{13}C$ couplings in **A**: 2-*C*-methylerythritol (analysed as tetraacetylated derivative) and **B**: Isoprenic units of dihydromenaquinones represented by the carbon skeleton of IPP.

In conclusion, the labelling experiments clearly revealed the biosynthesis of methylerythritol from a C_3 subunit (GAP) and a C_2 subunit (derived from pyruvate decarboxylation). Its branched C_5 skeleton results from the rearrangement of a straight chain C_5 precursor, much like the formation of the isoprenic units from the prenyl chains of the dihydromenaquinones in the same bacterium. According to the obvious resemblance between the formation of methylerythritol and the biosynthesis of isoprenic units via the GAP/pyruvate pathway, methylerythritol should be considered as an hemiterpene and as a possible biosynthetic precursor for IPP. This hypothesis is already supported by our preliminary results on the successful incorporation of deuterium labelled DL-methylerythritol into ubiquinone-8 of *E. coli* (Duvold *et al.*, unpublished results).

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(9) The 12 eV mass spectrum of the mixture of dihydromenaquinones showed molecular ions at m/e 718.6 and 786.6 for MK-8 (II-H_2) and MK-9 (II-H_2) respectively in a ca. 6:1 ratio. Ions m/z 135 and 136 corresponded to the fragment of the two last isoprenic units of the prenyl chain. Further fragmentation of isoprenic units by fission through the diallylic bonds were represented by the sizeable ions at m/e 135+n68 and 136+n68. Interruption of this sequence after the last possible diallylic fission revealed the location of the saturation.[10] Interruption of this sequence occured after m/e 340.3 for MK-8 (II-H_2) and after m/e 408.2 for MK-9 (II-H_2) which means that the saturation in both cases was located in the second isoprenic unit counting out from the aromatic ring (Fig. 2).

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(11) *C. ammoniagenes* (DSM 20305) was grown at 30°C for 24 h in a complex culture medium (1 l) containing peptone (10 g), yeast extract (3 g), NaCl (5 g), pH 7.1. Benzylviologen hydrochloride (50 mg) was added after 24 h growth, and the cells were further grown for 24 h. Cells were harvested by centrifugation and freeze dried (ca 1.4 g dry weight). The methylerythritol cyclodiphosphate was extracted by refluxing for 1h in EtOH/H_2O (1:1, v/v, 150 ml). The extract was evaporated under reduced pressure, redissolved in water (10 ml) and freeze-dried. The resulting powder was treated with HF (48%, 2 ml) for 24 h at r.t. in order to cleave the diphosphate ester linkages, then neutralised with NaOH and freeze-dried. The oily residue was acetylated (pyridine/Ac_2O, 1:1, v/v, 10 ml) for 3h at r.t. Solvents were removed under reduced pressure, and the crude mixture was purified by flash column chromotography (hexane/EtOAc 1:1) to yield impure methylerythritol triacetate. The triacetate fractions were collected and reacetylated (pyridine/Ac_2O, 1:1, v/v, 4 ml) overnight at 60 °C affording predominantly the corresponding tetraacetate. Purification by preparative TLC (toluene/EtOAc 4:1, two runs, R_f 0.38) afforded pure tetraacetate (2.8 mg). The cell residues after EtOH/H_2O extractions was reextracted for 45 min with refluxing $CHCl_3$/MeOH (2:1, v/v, 4x50 ml). The crude extract was brought to dryness and washed with hexane (4x10 ml). The hexane extract was filtered through a short plug of silica (CH_2Cl_2). Purification by TLC (CH_2Cl_2/hexane, 3:2) afforded an inseparable mixture of MK-8 (II-H_2) and MK-9 (II-H_2) (R_f 0.61, 2.2 mg). Methylerythritol tetraacetate was identified by comparison with a synthetic reference of DL-methylerythritol tetraacetate (Duvold *et al.*, unpublished results), the ^{1}H-NMR spectrum was in full accordance with published spectra.[8] The following ^{13}C NMR assignments are supported by supplementary experiments including DEPT, HMQC and HMBC. Methylerythritol tetraacetate (50 MHz, $CDCl_3$): 17.43 (C-5), 20.80 (-CO<u>C</u>H_3, 3x), 22.03 (-CO<u>C</u>H_3), 62.51 (C-4), 63.41 (C-1), 70.55 (C-3), 81.20 (C-2), 169.66 (-<u>C</u>OCH$_3$, 2x), 170.41 (-<u>C</u>OCH$_3$), 170.75 (-<u>C</u>OCH$_3$). MK-8/9 (II-H_2) (125 MHz, $CDCl_3$): 12.72 (3-Me), 15.99, 16.04 and 16.06 (C-5', units 3-7/8), 16.34 (C-5', unit 1), 17.72 (C-5', 8/9), 19.63 (C-5', unit 2), 25.30 (C-1', unit 2), 25.50 (C-1', unit 3), 25.73 (C-4', units 8/9), 26.04 (C-1', unit 1), 26.72-26.81, (C-1', units 4-8/9), 32.35 (C-3', unit 2), 36.60 (C-2', unit 2), 37.12 (C-4', unit 2), 39.80 (C-4', units 3-8/9), 40.07 (C-4', unit 1), 118.88 (C-2', unit 1), 124.31 (C-2', units 4-7/8), 124.45 (C-2', unit 8/9), 124.88 (C-2', unit 3), 126.23 and 126.35 (C-5 and C-8), 131.28 (C-3', unit 8/9), 132.18 and 132.24 (C-9 and C-10), 133.33 and 133.39 (C-6 and C-7), 134.67, 134.92 and 134.96 (C-2', units 4-7/8), 137.99 (C-3', unit 1), 143.39 (C-3), 146.26 (C-2), 184.59 and 185.53 (C-1 and C-4). Isotopic abundances were determined as previously described by comparison with an authentic non-labelled reference recorded under the same experimental conditions.[3a] For methylerythritol tetraacetate, the calculated isotopic abundances were first based on the signals of the acetate methyl groups and further on those of the supposed non-labelled carbon atoms of the C_5 skeleton (C-1, C-2, C-3) as reference signals. The values of the enriched carbon atoms in Fig. 3 are therefore given as mean values using these different carbon atoms as references. For MK-8/9 (II-H_2), the calculated isotopic abundances were based on the 3-Me carbon as reference carbon atom which, according to the biosynthesis of the quinone moiety, should not be labelled neither from C-1 nor C-6 of glucose. The values for isotopic abundances given for IPP in Fig. 3 are given as mean values for isoprenic units 1, 2, 3 and terminal.

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