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5-Hydroxypentane-2,3-dione (Laurencione), a Bacterial Metabolite of 1-Deoxy-D-*threo*-pentulose

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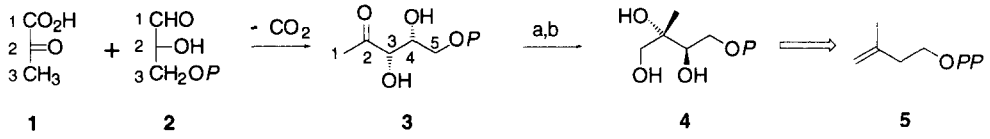
Abstract. Cell-free systems from the bacteria *Escherichia coli* and *Klebsiella planticola* that were incubated with ^{13}C labeled pyruvate and D-glyceraldehyde synthesized 5-hydroxypentane-2,3-dione (laurencione) along with 1-deoxy-D-*threo*-pentulose (1-deoxy-D-xylulose). Both compounds showed identical labeling patterns, indicating that the C_5 skeletons were derived from the condensation of (hydroxyethyl)thiamin on D-glyceraldehyde. Conversion of [5,5-$^2H_2$]deoxyxylulose into laurencione by a cell-free system from *E. coli* showed that the α-dione is obtained from the pentulose by water elimination.

A mevalonate-independent pathway for the biosynthesis of isopentenyl diphosphate **5**, the universal isoprenoid precursor, starting from pyruvate **1** and D-glyceraldehyde 3-phosphate **2** (Fig. 1),[1] is found in bacteria, algae and higher plants.[2] 1-Deoxy-D-*threo*-pentulose 5-phosphate **3** (1-deoxy-D-xylulose 5-phosphate) is the first C_5 precursor[3a] and was already known as a precursor of thiamin[4] and pyridoxol.[5] The gene of the thiamin diphosphate dependent synthase which catalyzes the formation of deoxyxylulose 5-phosphate was cloned from *Escherichia coli* and peppermint and overexpressed in *E. coli*,[6] allowing the preparative scale production deoxyxylulose with multiple ^{13}C labeling.[3c] A rearrangement, first detected by incorporation of glucose isotopomers with multiple ^{13}C labeling,[1,2b] yields from the straight-chain pentulose the branched skeleton of 2-*C*-methyl-D-erythritol 4-phosphate **4**. The branched carbon skeleton of 2-*C*-methyl-D-erythritol is formed in *Corynebacterium ammoniagenes* from pyruvate and glyceraldehyde by the same condensation and rearrangement as those involved in the formation of the isoprenic units of the dihydromenaquinone.[7a] Furthermore, deuterium labeled 2-*C*-methyl-D-erythritol was incorporated into ubiquinone and menaquinone of *E. coli*.[7b] Apparently, no degradation of the carbon skeleton and no changes of the oxidation state of carbon atoms C-1 and C-4 occurred. This suggested that 2-*C*-methyl-D-erythritol or rather its 4-phosphate are possible isoprenoid precursors or at least closely related to isoprenoids. If these compounds are not involved in other metabolic pathways, then the reaction catalyzed by the recently reported reducto-isomerase, converting deoxyxylulose 5-phosphate into methylerythritol 4-phosphate,[8] would represent the first committed step of this biosynthetic route.[7] No other intermediates are known.



1 2 3 4 5

Fig. 1. Mevalonate-independent route for isoprenoid biosynthesis: a) rearrangement; b) reduction by NADPH.

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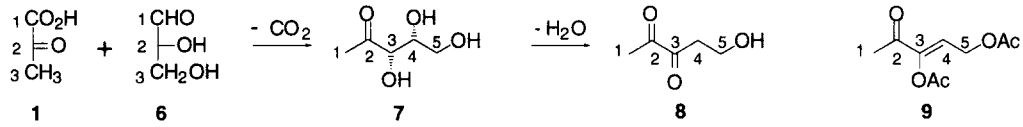
Formation of laurencione from pyruvate and D-glyceraldehyde

When unlabeled pyruvate **1** and D-glyceraldehyde **6** were incubated with crude or partially purified cell-free systems prepared from a wild type strain of *E. coli*, from the transformed *E. coli* strain pTAC-DXS, where deoxyxylulose 5-phosphate synthase was overexpressed,[6b] and from *Klebsiella planticola*, the main product deoxyxylulose **7** was always accompanied by another compound which was identified here as laurencione **8** (Fig. 2).[9-11] Separation of free deoxyxylulose **7** and laurencione **8** resulted often in unsatisfactory yields and was only attempted when purified samples of deoxyxylulose were required.[3c] Furthermore, according to the literature, significant losses by dimerization were expected for laurencione from silica gel chromatography of free laurencione.[9,12] Laurencione was therefore analyzed as the diacetylated derivative **9**. A pure sample of **9** obtained from the*K. planticola* cell-free system was isolated by TLC.[11] Its NMR (^1H and ^{13}C) and mass spectra and its GC retention time were identical with those of a reference sample obtained by $NaIO_4/RuO_2$ oxidation[13] of the acetate of commercial 3-pentyn-1-ol followed by acetylation or with those reported in the literature.[9,12] All characteristic features were also found in the spectra of the samples obtained from *E. coli* strains for which the deoxyxylulose triacetate and the diacetylated derivative **9** of laurencione were not separated.

The origin of carbon atoms of laurencione was determined from labeling experiments performed with cell-free systems obtained from *E. coli* pTAC-DXS, using either [2-^{13}C]pyruvate and [1-^{13}C]-DL-glyceraldehyde,[14a] [2-^{13}C]pyruvate[14c] or [3-^{13}C]pyruvate[14b] and unlabeled D-glyceraldehyde, or unlabeled pyruvate and [2-^{13}C]-DL-glyceraldehyde.[14d] Carbon atoms C-1 and C-2 of laurencione **8** respectively corresponded to C-3 and C-2 of pyruvate **1**, and C-3 and C-4 of laurencione **8** to C-1 and C-2 of D-glyceraldehyde **6** (Fig. 2).



1 **6** **7** **8** **9**

Fig. 2. Biosynthesis of laurencione **8** from pyruvate **1** and glyceraldehyde **6** and from 1-deoxy-D-xylulose **7** by bacterial cell-free systems.

Conversion of 1-deoxy-D-xylulose into laurencione

The labeling patterns obtained from the incorporation of ^{13}C labeled pyruvate and D-glyceraldehyde were identical with those observed for deoxyxylulose in the same feeding experiments, indicating that both C_5 carbon skeletons most probably resulted from the same condensation reaction (Fig. 2). Synthetic [5,5-$^2H_2$]deoxyxylulose[15] was converted into [5,5-$^2H_2$]laurencione[14e] (identified by ^1H- and ^{13}C-NMR spectroscopy) by a cell-free system from *E. coli*. In the deuterium labeled **9**, the signal at 4.75 ppm of the two C-5 protons replaced by deuteriums was missing, and the signal of the C-4 proton appeared as a singlet at 6.45 ppm instead of a triplet. The presence of two deuterium atoms at C-5 was further corroborated by the absence in the ^{13}C-NMR spectrum of the signal of the C-5 carbon atom due the presence of deuterium in the α position and by an upfield shift ($\Delta\delta$=-0.3 ppm) of the signal of C-4 due to the deuteriums in the β-position. The incorporation of labeled deoxyxylulose into laurencione proved a precursor-to-product relationship between the two compounds and shed light on a novel dehydrase activity catalyzing the elimination of water from deoxyxylulose (Fig. 2). A similar diol/ketone conversion by elimination of a proton in α-position of a carbonyl group is for instance found in the conversion of 2-keto-3-deoxygluconate 6-phosphate from gluconate 6-phosphate, a key reaction in glucose catabolism via the Entner-Doudoroff pathway in bacteria.

On the putative role of laurencione

Free laurencione is a natural product accumulated in the red alga *Laurencia spectabilis*,[9] and its dimer was isolated from *Laurencia pennatifida*.[16] Laurencione was proposed as a potential isoprenoid precursor via 3,5-hydroxypentan-2-one.[15] The branched isoprenic skeleton could however directly result from laurencione via a benzilic acid-type rearrangement resembling for instance that involved in the Cannizzaro reaction catalyzed by the glyoxalase I (EC 4.4.1.5), the hydride shift being replaced by the transfer of an alkyl group (Fig. 3). This hypothesis cannot be excluded, but is in contradiction with our previous results concerning the incorporation of deuterated 2-*C*-methyl-D-erythritol into isoprenoids,[7b] as no evident biogenetic links can be found between laurencione and this tetrol.

Laurencione was proposed as a precursor of pyridoxol.[17] It fits perfectly into the hypothetical biogenetic scheme proposed for thiamin biosynthesis in facultative anaerobic prokaryotes and in plants.[18] For the formation of the thiazole moiety, water elimination was proposed to occur on the Schiff base corresponding to the adduct between deoxyxylulose and L-tyrosine. The same Schiff base could be directly obtained from the formation of L-tyrosine and laurencione which results from deoxyxylulose by water elimination. Deuterium-labeled laurencione has been synthesized, and experiments are being performed with incorporation into bacteria and plants in order to investigate its possible role in the metabolism of isoprenoids and thiamin.

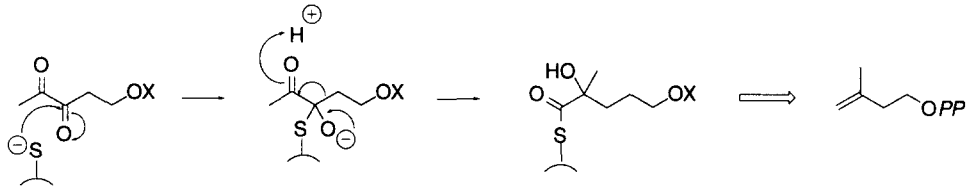


Fig. 3. Hypothetical biogenetic scheme for the formation of the isoprenic skeleton from laurencione (X = H or phosphate).

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10. ^{13}C labeled compounds, cultures of *E. coli* wild type strain E15 and transformed pTAC-DXS strain, culture of *K. planticola* IAM 1133 (Institute of Molecular and Cellular Biosciences, the University of Tokyo, Japan) and preparation of the cell-free systems were as previously described.[3c,6b] Changes are indicated below.
Enzymatic formation of unlabeled laurencione. Cell-free systems (20 ml) from both *E. coli* strains (0.3 g, lyophilized cells) were incubated in presence of unlabeled sodium pyruvate (50 mg, 0.45 mmol) and D-glyceraldehyde (40 mg, 0.45 mmol) for 20h at 37°C on an orbital shaker. Reaction was stopped by heating at 80°C for 5 min. Denatured proteins were removed by centrifugation (18000 g). The supernatant was lyophilized, and the residue directly acetylated using acetic anhydride/pyridine (1:1, 10 ml, overnight at room temperature). In the case of the incubations with the *E. coli* strains, the mixture of acetylated deoxyxylulose and laurencione (24 mg) was purified by TLC (hexane/EtOAc, 1:1, R_f=0.45). For the incubation with *K. planticola*, pure diacetylated derivative of laurencione was obtained from the preceding mixture after repeated TLC (toluene/EtOAc, 8:2, 3 migrations).
[2,3-$^{13}C_2$]Laurencione. The cell-free system (20 ml) prepared from freeze-dried *E. coli* pTAC-DXS (0.6 g) was incubated with sodium [2-^{13}C]pyruvate (1.8 mmol, 199 mg) and [1-^{13}C]-DL-glyceraldehyde (1.8 mmol, 162 mg) as indicated above. The residue obtained after lyophilization of the protein-free incubation system was extracted with methanol (3x25 ml). After evaporation of the solvent, the crude fraction yielded by TLC ($CHCl_3/CH_3OH$, 80:20) a mixture of deoxyxylulose and laurencione (R_f=0.40, 75 mg). An aliquot (30 mg) was acetylated, and TLC (diethyl ether/pentane, 4:6, 2 migrations) afforded the diacetylated derivative of laurencione (R_f=0.40, 8 mg) and the triacetate of deoxyxylulose (R_f=0.33, 22 mg).
Other ^{13}C labeled laurencione samples. All other incubations of labeled material were performed as described above with cell-free systems (10 ml) prepared from fresh cells of *E. coli* pTAC-DXS (ca. 0.5 g) using either sodium [3-^{13}C]pyruvate (50 mg) and unlabeled D-glyceraldehyde (40 mg), [2-^{13}C]pyruvate (50 mg) and unlabeled D-glyceraldehyde (40 mg), unlabeled sodium pyruvate (50 mg) and [2-^{13}C]-DL-glyceraldehyde (40 mg), or [5,5-$^2H_2$]deoxyxylulose (50 mg, 0.37 mmol), respectively yielding after acetylation and TLC (diethyl ether/pentane, 4:6, Rf=0.25) the mixture of the acetylated derivatives of [1-^{13}C]deoxyxylulose and [1-^{13}C]laurencione (2.3 mg), [2-^{13}C]deoxyxylulose and [2-^{13}C]laurencione (2.5 mg), [4-^{13}C]deoxyxylulose and [4-^{13}C]laurencione (2.2 mg) and [5,5-$^2H_2$]deoxyxylulose and [5,5-$^2H_2$]laurencione (7.0 mg, 1:3). According to GC and NMR spectroscopy, laurencione/deoxyxylulose ratio was between 1:6 and 1:3 depending on the sample.
11. Diacetylated derivative **9** of laurencione from *K. planticola*. ^1H-NMR: δ (ppm) = 2.09 (3H, s, CH_3COO-), 2.26 (3H, s, CH_3COO-), 2.32 (3H, s, 1-H), 4.73 (2H, d, $J_{4,5}$=6.2Hz, 2x5-H), 6.46 (1H, t, $J_{4,5}$=6.3Hz, 4-H). ^{13}C-NMR: δ (ppm) = 20.3 (**CH_3**COO-), 20.6 (**CH_3**COO-), 25.2 (C-1), 58.6 (C-5), 125.0 (C-4) 147.1 (C-3), 168.3 (CH_3**CO**O-), 170.3 (CH_3**CO**O-), 190.9 (C-2). GC-MS (chemical ionization using isobutane as reactant gas): *m/z* (%) = 201 ($[M+H]^+$, 8%), 158 (M^+-CH_2CO, 3%), 141 (201-AcOH, 100%), 116 (M^+-2xCH_2CO, 1%). These spectroscopic data were identical with those of **9** obtained by synthesis or from cell-free systems of the two *E. coli* strains. ^{13}C assignments from the literature[9] had to be revised.
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14. (a) [2,3-$^{13}C_2$]-**9**. ^1H-NMR: δ (ppm) = 2.10 (3H, s, CH_3COO-), 2.27 (3H, s, CH_3COO-), 2.34 (3H, dd, $^3J_{1\text{-}H,3\text{-}C}$=1.4Hz, $^2J_{1\text{-}H,2\text{-}C}$=6.3Hz, 1-H), 4.74 (2H, ddd, $J_{4,5}$=6.0Hz, $^3J_{5\text{-}H,3\text{-}C}$=5Hz, $^4J_{5\text{-}H,2\text{-}C}$=1Hz, 2x5-H), 6.47 (1H, dxquadr, $J_{4,5}$=$^3J_{4,2\text{-}C}$=6.4Hz, $^2J_{4\text{-}H,3\text{-}C}$=3.4Hz, 4-H). ^{13}C-NMR: δ (ppm) = 147.0 (C-3, d,$^1J_{2,3}$=61Hz), 190.9 (C-2, d, $^1J_{2,3}$=58Hz).
(b) [1-^{13}C]-**9** (not separated from [1-^{13}C]deoxyxylulose triacetate). ^1H-NMR: δ (ppm) = 2.10 (3H, s CH_3COO-), 2.27 (3H, s CH_3COO-), 2.34 (3H, d, $^1J_{1\text{-}H,1\text{-}C}$=129Hz, 1-H), 4.74 (2H, d, $J_{4,5}$=6.0Hz, 2x5-H), 6.47 (1H, t, $J_{4,5a}$=6.2Hz, 4-H). ^{13}C-NMR: δ (ppm) = 25.2 (C-1).
(c) [2-^{13}C]-**9** (not separated from [2-^{13}C]deoxyxylulose triacetate). ^1H-NMR: δ (ppm) = 2.09 (3H, s, CH_3COO-), 2.27 (3H, s, CH_3COO-), 2.34 (3H, d, $^2J_{1\text{-}H,2\text{-}C}$=6.2Hz, 3x1-H), 4.74 (2H, d, $J_{4,5}$=6.4Hz 2x5-H), 6.47 (^1H, dt, $J_{4,5}$=6.4Hz, $^3J_{4\text{-}H,2\text{-}C}$=3.5Hz, 4-H). ^{13}C-NMR: δ (ppm) = 190.9 (C-2).
(d) [4-^{13}C]-**9** (not separated from [4-^{13}C]deoxyxylulose triacetate). ^1H-NMR: δ (ppm) = 2.09 (3H, s, CH_3COO-), 2.27 (3H, s, CH_3COO-), 2.34 (3H, s, 1-H) 4.74 (2H, dd, $J_{4,5}$=6.4Hz, $^2J_{5\text{-}H,4\text{-}C}$=5.2Hz, 2x5-H), 6.47 (1H, dt, $J_{4,5}$=6.4Hz $^1J_{4\text{-}H,4\text{-}C}$=163Hz, 4-H). ^{13}C-NMR: δ (ppm) = 125.0 (C-4).
(e) [5,5-$^2H_2$]-**9** (not separated from [5,5-$^2H_2$]deoxyxylulose triacetate). ^1H-NMR: δ (ppm) = 2.09 (3H, s, CH_3COO-), 2.27 (3H,s, CH_3COO-), 2.33 (3H, s, 1-H), 6.45 (1H, s, 4-H). ^{13}C-NMR: δ (ppm) = 20.2 (**CH_3**COO-), 20.6 (**CH_3**COO-), 25.2 (C-1), 124.8 (C-4), 147.0 (C-3), 190.1 (C-2); signals of acetoxy carbonyl groups are overlapping with those of deoxyxylulose triacetate.
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