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Biosynthesis of Menaquinone in *E. coli*: Identification of an Elusive Isomer of SEPHCHC

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In the biosynthesis of menaquinone in bacteria, the thiamine diphosphate-dependent enzyme MenD catalyzes the decarboxylative carboligation of α-ketoglutarate and isochorismate to (1*R*,2*S*,5*S*,6*S*)-2-succinyl-5-enolpyruvyl-6-hydroxycyclohex-3-ene-1-carboxylate (SEPHCHC). The regioisomer of SEPHCHC, namely (1*R*,5*S*,6*S*)-2-succinyl-5-enolpyruvyl-6-hydroxycyclohex-2-ene-1-carboxylate (iso-SEPHCHC), has been considered as a possible product, however, its existence has been doubtful due to a spontaneous elimination of pyruvate from SEPHCHC to 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (SHCHC). In this work, the regioisomer iso-SEPHCHC was distinguished from SEPHCHC by liquid chromatography-tandem mass spectrometry. Iso-SEPHCHC was purified and identified by NMR spectroscopy. Just as SEPHCHC remained hidden as a MenD product for more than two decades, its regioisomer iso-SEPHCHC has remained until now.

Introduction

The metabolite chorismate, which results from the shikimate biosynthetic pathway, represents a central branching point and, as such, is the precursor of numerous aromatic natural products as well as some nonaromatic secondary metabolites in microorganisms and plants.[1] Thus, in anaerobic and Gram-positive bacteria, the biosynthesis of the electron carrier menaquinone begins with the conversion of chorismate into isochorismate (**1**). Subsequently, the thiamine diphosphate (ThDP)-dependent enzyme (1*R*,2*S*,5*S*,6*S*)-2-succinyl-5-enolpyruvyl-6-hydroxycyclohex-3-ene-1-carboxylate (SEPHCHC)-synthase MenD catalyzes the Stetter reaction (1,4-addition) of α-ketoglutarate (**2**) and isochorismate (**1**) into SEPHCHC (**3**) (Scheme 1).[2] SEPHCHC (**3**) is then transformed into 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (SHCHC, **4**) by the enzyme MenH.[3]

It has long been believed that SHCHC (**4**), instead of SEPHCHC (**3**), was the physiological product of MenD.[4] SEPHCHC (**3**) is an unstable compound that rapidly undergoes a base-catalyzed (or spontaneous) elimination of the enolpyruvate moiety into SHCHC (**4**).[2,4] In a breakthrough publication by Jiang et al., kinetic studies allowed to differentiate the rates between isochorismate (**1**) consumption and SHCHC (**4**) formation, while spectroscopic characteristics of SHCHC (**4**) and SEPHCHC (**3**) were in consistence with the re-evaluation of MenD activity, being SEPHCHC (**3**) the enzymatic product.[2] Shortly thereafter, MenH was discovered.[3]

To obtain an alternative substrate for MenD that is more stable than the natural substrate isochorismate, analogue **5**, in which the enolpyruvyl substituent is replaced by a carboxymethoxyl group, has been synthesized by Fang et al. (Scheme 2).[5] The enzymatic product **6** [from **5** and α-ketoglutarate (**2**)] undergoes spontaneous isomerization to the more stable conjugate iso-**6**. The increased stability of iso-**6** is caused by the shift of the ring methylene group, which leads to an α,β-unsaturated carbonyl between the chain originating from ketoglutarate and the C6 of the isochorismate-derived ring.[5]

Previously, we reported on the microbial access to cyclic derivatives of SEPHCHC by exploiting the substrate promiscuity of MenD.[6] Rationally designed MenD variants were used for the *in vivo* conversion in *E. coli* of the isochorismate analogues (5*S*,6*S*)-5,6-dihydroxycyclohexa-1,3-diene-1-carboxylate (2,3-*trans*-CHD, **7**) to (1*R*,2*S*,5*S*,6*S*)-2-succinyl-5,6-dihydroxycyclohex-3-ene-1-carboxylate (SDHCHC, **8**); and (5*S*,6*S*)-6-amino-5-hydroxycyclohexa-1,3-diene-1-carboxylate (2,3-*trans*-CHA, **9**) to 2-succinyl-6-amino-5-hydroxycyclohex-3-ene-1-carboxylate (SAHCHC, **10**) (Scheme 2). As expected, the products of the MenD-catalyzed reactions isomerized analogously to compound

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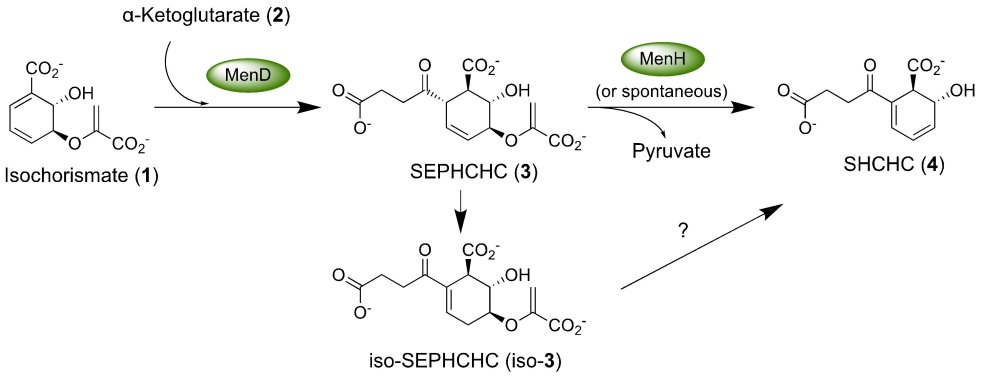
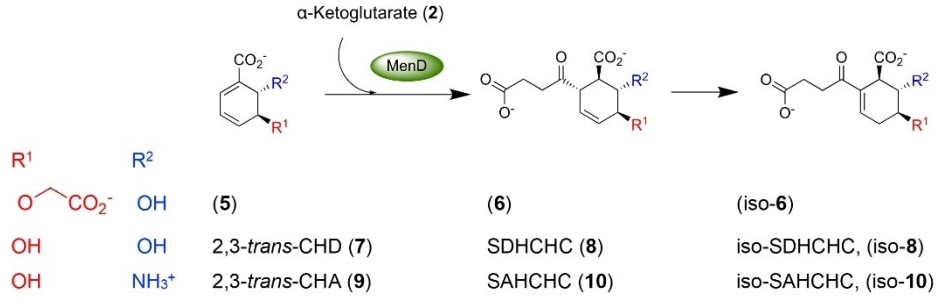


Scheme 1. First committed step towards the biosynthesis of menaquinone in *E. coli*, and subsequent reactions from SEPHCHC.



Scheme 2. Spontaneous isomerization of the isochorismate analogues (**6**),[5] 2,3-*trans*-CHD (**7**), and 2,3-*trans*-CHA (**9**).[6]

6, resulting in the regioisomers iso-SDHCHC (iso-**8**) and iso-SAHCHC (iso-**10**).[6]

However, it has been suggested that a similar product arising from the isochorismate-derived product SEPHCHC (**3**), namely iso-SEPHCHC (iso-**3**), is not observed because SEPHCHC undergoes spontaneous elimination to form SHCHC (**4**) (Scheme 1).[5] Accordingly, no reports on the isomerization of SEPHCHC have been published as yet. Herein, we report that the physiological enzymatic product SEPHCHC (**3**) isomerizes nonenzymatically to the more stable regioisomer iso-**3**.

Results and Discussion

In liquid chromatography coupled with mass spectrometry (LC–MS) analysis of *E. coli* fermentation broth, we observed that the peak corresponding to the *m/z* value 327.2 of SEPHCHC had a shoulder or even the occurrence of two separate peaks, depending on the fermentation conditions (Figure 1A). For these reasons, we hypothesized that SEPHCHC (**3**) exists as a mixture of isomers.

To clarify the identity of the presumed isomers, LC–MS/MS analyses of **3** and the tentative iso-**3** peaks were performed using multiple reaction monitoring (MRM) scans. Both peaks showed transition ions at *m/z* 327.2→238.9, 133.2, and 87.0; however, the ratio of the intensity of the signals at *m/z* 87.0, *m/z* 133.2, and *m/z* 238.9 was different (Figure 2).

We performed combined chromatographic, spectrometric, and spectroscopic studies to structurally characterize the putative new isomer. Purification of iso-**3** was performed by anion exchange chromatography, solvent extraction, and reversed-phase chromatography of the fermentation broth. In all steps, the pH was kept neutral or acidic to prevent base-catalyzed reactions of **3** and iso-**3** into **4**. The compound detected at a retention time $t_R = 7.2$ min (Figure 1A) was identified as iso-SEPHCHC (iso-**3**) (Scheme 1) after purification (Figure 1B, $t_R = 7.0$ min, peak slightly shifted due to the sample matrix) and subsequent analysis by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. Here, almost all signals in the proton spectrum of iso-**3** matched those recorded for **3**, except for two signals at 2.40–2.50 and 2.80–2.92 ppm, and only one instead of two olefinic protons (Figure S3). The presence of these signals underlined the differences in regiochemistry with respect to the ring methylene group and indicated the formation of an α,β-unsaturated carbonyl in iso-**3**, as we have reported for iso-**8** and iso-**10**.[7,6]

To confirm the existence of both regioisomers, purified isochorismate (**1**)[8] was incubated *in vitro* with purified MenD for 10 minutes and the reaction products were analyzed by LC–MS/MS, monitoring the transition ions with *m/z* 327.1→87.1, *m/z* 327.1→133.2, and *m/z* 327.1→238.9. Two peaks corresponding to **3** and iso-**3** were clearly distinguished (Figure 1C). The observed differences in product ratios between **3** and iso-**3** *in vivo* (1:1) and *in vitro* (4:1) could be due to experimental bias, e.g., different pH or ionic strength of the fermentation

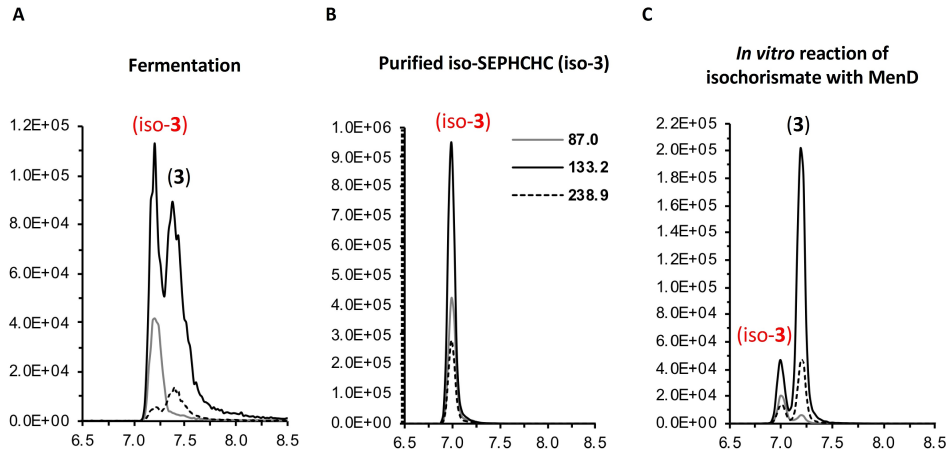


Figure 1. LC–MRM scans of the transition ions with *m/z* 327.2→87.0, 133.2, and 238.9. (A) Culture broth of *E. coli* F97/*pC20-menD*. (B) Purified iso-SEPHCHC (iso-**3**). (C) *In vitro* reaction of isochorismate (**1**) with MenD.

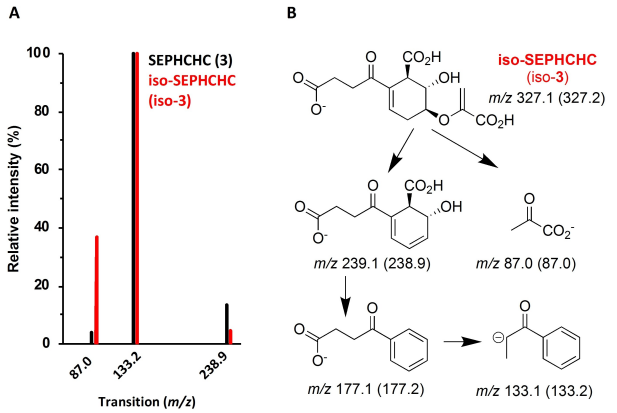


Figure 2. Differential fragmentation pattern of iso-SEPHCHC (iso-**3**) and SEPHCHC (**3**). (A) Intensity of the transition ions with *m/z* 87.0, 133.2, and 238.9 released from the parent ion with *m/z* 327.2. (B) Proposed fragmentation of purified iso-SEPHCHC (iso-**3**).

broth and the *in vitro* reaction buffer. Alternatively, the cytosolic environment of MenD during fermentation in engineered *E. coli* cells may affect the tertiary structure of MenD, resulting in subtle changes in the orientation of the active site residues when compared to MenD used *in vitro*. The impact of small changes in the active site for product formation was previously highlighted when the importance of two conserved arginine residues and the formation of an alternative binding site for the ThDP-bound post-decarboxylation intermediate was observed.[9] Moreover, the rational design of MenD variants and an isochorismate analog (**5**) support this hypothesis, as both showed the presence of the more stable iso-forms – namely iso-**6**, iso-**8**, iso-**10** – *in vivo* in larger yields.[5–7] Finally, we cannot rule out the possibility that allosteric or other regulatory effects due to the presence or absence of biosynthetic downstream metabolites influence the formation of iso-**3**, as has been reported for 1,4-dihydroxy-2-naphthoic acid, which limits metabolic flux into the menaquinone pathway by inhibiting MenD.[10]

Conclusion

We present here experimental evidence of the isomerization of SEPHCHC (**3**) into iso-SEPHCHC (iso-**3**), in addition to spontaneous or enzyme-catalyzed pyruvate elimination into SHCHC (**4**) (Scheme 1). This suggests that iso-**3** exists as a second stable metabolite and constitutes a new member of the already broad and eponymous chorismate-derived branching tree. In other words, iso-SEPHCHC has been an elusive regioisomer of SEPHCHC. In light of our results and mechanistic considerations, it can be assumed that MenH also accepts iso-**3** as a substrate. This finding opens up new perspectives for understanding the supposedly well-known menaquinone biosynthesis.

Experimental section

For full experimental details, see the Supporting Information (SI) text.

Strain, cultivation and purification of iso-3: The modified strain *E. coli* F97 [genotype LJ110 *lac*::(P_{tac}-*aroFBL*+) Δ(*pheA tyrA aroF*) Δ(*entCEBA*) Δ*menC*] transformed with *pC20-menD*[6] was used for *in vivo* conversions. The cultures were centrifuged, the supernatant was filtered and loaded onto an anion-exchange column (Dowex 1X4, 200 mL, 20–50 mesh). Then, 1.5 column volumes (CV) of 2 M NH_4Cl was added and incubated for 20 min, and a further 2 CV of 2 M NH_4Cl was added. The fractions containing **3** and iso-**3** were identified by MS, pooled (200 mL), and acidified. Hydrophobic impurities were removed by extracting with dichloromethane and ethyl acetate. The solvent in the aqueous phase was removed under reduced pressure at 25 °C, followed by freeze-drying. The sample was dissolved in H_2O, and further purified by gel filtration (Sephadex G10 packed in an XK 26/20 column, Amersham Biosciences) using an Äktaprime Plus system (GE Healthcare Life Sciences). The fractions containing **3** and iso-**3** were pooled, freeze-dried and dissolved in diluted formic acid (2–3%). The final purification step was preparative reversed-phase chromatography using an Agilent 1200 HPLC system with a Multospher column (100 RP C18 5 µm, 250×20 mm, CS-Chromatographie). An elution gradient of 0.1% formic acid and acetonitrile was applied. The fractions containing **3** and iso-**3**, respectively, were pooled,

acetonitrile was removed under reduced pressure and the remainder was freeze-dried (yield: 8 mg per 400 mL culture).

Enzymatic assays: MenD was produced and purified as described before.[6] The enzymatic reactions were performed at 28 °C with 6 mM α-ketoglutarate, 1 mM isochorismate and 0.2 mg/mL MenD using 50 mM Tris-HCl buffer pH 7.4, containing 200 mM NaCl, 1 mM $MgCl_2$ and 50 μM thiamine diphosphate. Isochorismate was obtained as described before.[8]

HPLC-MS: Samples were acidified with 1% formic acid, centrifuged (1 min, 19600×*g*), filtered (0.2 μm cellulose acetate filter, CS-Chromatographie) and diluted 1:40 with H_2O. The RP-HPLC-MS analysis was performed using an Agilent 1100 HPLC system (Agilent Technologies) coupled to an API 2000 or API Qtrap 4500 triple quadrupole mass spectrometer (AB SCIEX) with a Multospher RP C18, AQ-3 μm EC column (60×2 mm, CS-Chromatographie). As mobile phase, a gradient of 0.1% (vol/vol) formic acid and acetonitrile was applied. The compounds were detected by mass spectrometry in negative ion mode using the parameters (API Qtrap 4500) listed in Table S2. MRM scans were performed using the parameters (API Qtrap 4500) and transition ions listed in Table S2 and S3.

Abbreviations: ^{1}H NMR: proton nuclear magnetic resonance; 2,3-*trans*-CHD: (5*S*,6*S*)-5,6-dihydroxycyclohexa- 1,3-diene-1-carboxylate; 2,3-*trans*-CHA: (5*S*,6*S*)-6-amino-5- hydroxycyclohexa-1,3-diene-1-carboxylate; LC–MS: liquid chromatography coupled with mass spectrometry; SAHCHC: 2-succinyl-6-amino-5-hydroxycyclohex-3-ene-1-carboxylate; SDHCHC: (1*R*,2*S*,5*S*,6*S*)-2-succinyl-5,6- dihydroxy-cyclohex-3-ene-1-carboxylate; SEPHCHC: (1*R*,2*S*, 5*S*,6*S*)-2-succinyl-5-enolpyruvyl-6-hydroxycyclohex-3-ene-1-carboxylate; SHCHC: 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate; ThDP: thiamine diphosphate.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: C–C coupling · chorismate · enzymes · Stetter reaction · thiamine · Umpolung

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