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# Alteration of the Route to Menaquinone towards Isochorismate-Derived Metabolites

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*Dedicated to the memory of Jonathan B. Spencer*

Chorismate and isochorismate constitute branch-point intermediates in the biosynthesis of many aromatic metabolites in microorganisms and plants. To obtain unnatural compounds, we modified the route to menaquinone in *Escherichia coli*. We propose a model for the binding of isochorismate to the active site of MenD ((1*R*,2*S*, 5*S*,6*S*)-2-succinyl-5-enolpyruvyl-6-hydroxycyclohex-3-ene-1-carboxylate (SEPHCHC) synthase) that explains the outcome of the native reaction with α-ketoglutarate. We have rationally designed variants of MenD for the conversion of several isochorismate analogues. The double-variant Asn117Arg–Leu478Thr preferentially converts (5*S*,6*S*)-5,6-dihydroxycyclohexa-1,3-diene-1-carboxylate (2,3-*trans*-CHD), the hydrolysis product of isochorismate, with a >70-fold higher ratio than that for the wild type. The single-variant Arg107Ile uses (5*S*,6*S*)-6-amino-5-hydroxycyclohexa-1,3-diene-1-carboxylate (2,3-*trans*-CHA) as substrate with >6-fold conversion compared to wild-type MenD. The novel compounds have been made accessible in vivo (up to 5.3 g $L^{-1}$). Unexpectedly, as the identified residues such as Arg107 are highly conserved (>94%), some of the designed variations can be found in wild-type SEPHCHC synthases from other bacteria (Arg107Lys, 0.3%). This raises the question for the possible natural occurrence of as yet unexplored branches of the shikimate pathway.

Chorismate is a branch-point intermediate of the shikimate pathway, and the major precursor of aromatic metabolites in microorganisms and plants.[1] L-Tryptophan, L-phenylalanine, L-tyrosine, ubiquinone, folate, catechol, and salicylate are synthesized from chorismate (**1**) and its derivatives isochorismate (**2**) and aminodeoxyisochorismate (ADIC, **3**).[2] Derivatives, such as cyclohexanecarboxylate, are utilized in microorganisms for the biosynthesis of secondary metabolites.[3,4] The shikimate pathway has been genetically engineered for the production of known natural products, for example, (5*S*,6*S*)-5,6-dihydroxycyclohexa-1,3-diene-1-carboxylate (2,3-*trans*-CHD; **4**) and (5*S*,6*S*)-6-amino-5-hydroxycyclohexa-1,3-diene-1-carboxylate (2,3-*trans*-CHA; **5**), themselves chiral building blocks useful for stereoselective synthesis.[5–7] Both compounds are known from a biosynthetic context[8] and have been produced by microbial fermentation with *Escherichia coli* strains (Scheme 1).[9]

In the physiological context of *E. coli*, chorismate (**1**) is converted into isochorismate (**2**) by the isochorismatases EntC and MenF. In the menaquinone (vitamin $K_2$) biosynthesis, thiamine diphosphate (ThDP) dependent MenD [(1*R*,2*S*,5*S*,6*S*)-2-succinyl-5-enolpyruvyl-6-hydroxycyclohex-3-ene-1-carboxylate (SEPHCHC) synthase from *E. coli*, as used throughout this work] catalyzes the carboligation of **2** and α-ketoglutarate (**6**) into SEPHCHC (**7**). MenD accepts mimetics of its physiological substrate **2**, such as **4**, forming (1*R*,2*S*,5*S*,6*S*)-2-succinyl-5,6-dihydroxycyclohex-3-ene-1-carboxylate (SDHCHC) (**8**) (Scheme 1).[10–12] However, further derivatives of **2**, most importantly 2,3-*trans*-CHA (**5**), have been assessed as substrates with no significant conversion by wild-type MenD.[11]

Despite this drawback, our aim was to make use of the inherent chemical diversity of **1** and **2** and their derivatives by rationally designed variants of MenD to enable transformation of **5**. Thereby, functionalized non-aromatic cyclic compounds should be accessible in vivo through metabolic engineering, thus extending the known network of the shikimate pathway to "unnatural" metabolites. Conversely, the generated compounds could act as inhibitors of the shikimate pathway, as this pathway itself and particularly menaquinone biosynthesis are primary targets for antibiotics against patho-

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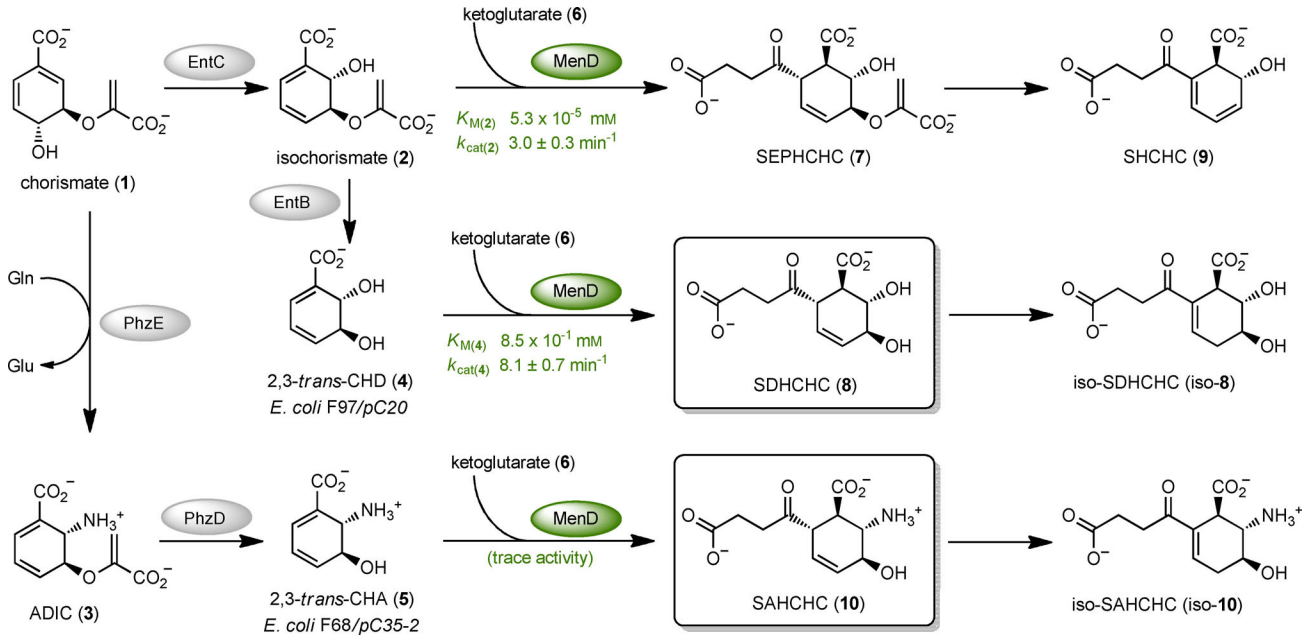


**Scheme 1.** Modification of natural pathways from chorismate (**1**) in *E. coli*. The kinetic parameters of purified MenD with isochorismate (**2**)[14] and 2,3-*trans*-CHD (**4**; this work) are shown in green.

genic strains such as *Staphylococcus* and *Mycobacterium tuberculosis*.

To gain (in vivo) access to SDHCHC (**8**), the 2,3-*trans*-CHD (**4**) overproducer *E. coli* F97/*pC20* was used as a host for the overexpression of MenD. This strain had been engineered for improved supply of **1**.[11] After the cultivation of *E. coli* F97/*pC20* (negative control) and *E. coli* F97/*pC20-menD* in shaking flasks, the culture broth was analyzed by LC-MS/MS (Table S4 in the Supporting Information). For *E. coli* F97/*pC20-menD*, a peak corresponding to the *m/z* value for **8** ($t_R$ = 5.0 min, *m/z* 256.85) was observed (Figure S1). Besides this, two minor peaks ($t_R$ = 3.9 and 6.6 min) with the same *m/z* and daughter ions were observed. The compound with $t_R$ = 3.9 min was purified and analyzed by NMR spectroscopy. The signals agree with the data reported for the regioisomer of **8**, iso-SDHCHC (iso-**8**; Scheme 1).[9,11]

Isochorismate (**2**) is both the physiological substrate of MenD and a precursor of **4**. The kinetic parameters of the purified MenD were determined for **4**: the catalytic efficiency ($k_{cat}/K_m$) was found to be $6\times10^3$ times lower than for **2** (Scheme 1).[13] As expected, there is a competition for the active site of MenD, and a peak corresponding to the physiological product SEPHCHC (**7**, $t_R$ = 7.2 min, *m/z* 327.1) was also observed. To increase the selectivity towards **4** versus **2** and to broaden its specificity towards **5** as a substrate, MenD had to be rationally optimized. MenD forms a dimer of dimers in solution. One dimer pair forms the active site, which consists mainly of a hydrophobic pocket and a highly basic patch.[15] Until now, efforts to obtain the *Ec*MenD structure with bound **2** have been unsuccessful.[16] Clues as to the binding of substrates derive from mutation studies using **2** and a substrate analogue in combination with kinetic parameters.[15,17,18]

To modify the substrate scope of MenD, we proposed a model for the binding of **2** that explains the outcome of the reaction. Furthermore, the following criteria were taken into account.

1) Intramolecular [3,3′]- and [1,5′]-sigmatropic rearrangements of isochorismate should be avoided.[19,20]
2) The distance of **2** to succinic semialdehyde-ThDP (formed by enzymatic decarboxylation of **6**) should be within the van der Waals radius and smaller than 3.7 Å.[21]
3) The model should be in accordance with the experimental data and kinetic parameters.
4) The binding of **2** to MenD might not differ from the binding of this substrate to enzymes other than MenD: The isochorismate synthase EntC and isochorismatase PhzD have been co-crystallized with **2** and served as a basis for the docking of **2** to MenD.[19]

In first place, the docking of the substrate isochorismate "as it is" did not yield a model according to the criteria stated above. For this reason, and according to the catalytic mechanism,[18,22] we assumed that a transition state is formed by an opening of the double bond of carbons C1 and C6 of **2**. This changes the hybridization of both carbons from $sp^2$ to $sp^3$, with an influence on the structure of the ring system. Docking studies with a transition-like conformation containing only one double bond in the ring system ("1,6-dihydroisochorismate") led to a model that explains the regio- and stereoselective reaction outcome with MenD (Figure 1). According to the proposed model, the cyclohexene ring of "1,6-dihydroisochorismate" lies above and parallel to the thiazole ring of ThDP. This hydrophobic part of the substrate is located in a hydrophobic

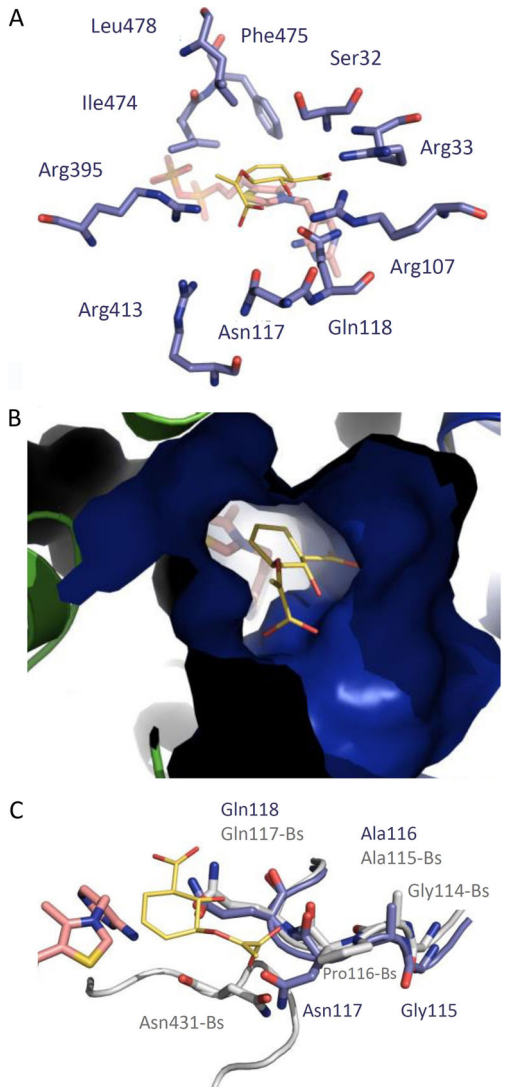


**Figure 1.** Docking model of *Ec*MenD (PDB ID: 3HWX) with "1,6-dihydroisochorismate" as a surrogate intermediate. A) View of the active center with "1,6-dihydroisochorismate". B) View of the substrate channel. C) Overlap of the active sites of *Ec*MenD and *Bs*MenD (PDB ID: 2X7J).

pocket interacting with Ile474, Leu478, and Phe475. By means of the repulsion of the carboxyl moiety, this pocket could prevent the sigmatropic rearrangement of **2** into isoprephenate.[18,19] Nucleophilic attack of the succinic semialdehyde-ThDP intermediate at C6 of **2** is thus reasonable. An electrophilic attack at C1 of **2** should also take place, to finalize the opening of the C1=C6 double bond. A conserved water molecule that lies between Ser32 and Gln118 could be responsible for this step, in analogy to the mechanism demonstrated for EntC.[19]

In our model, the C1-carboxyl and C6-hydroxy groups of "1,6-dihydroisochorismate" interact mainly with Arg107. Regarding the enolpyruvyl moiety, the side chain of Asn117 is situated next to the carboxylate and Leu478 accommodates the methylene moiety (Figure 1 A). The substrate channel matches exactly the shape of the enolpyruvyl moiety (Figure 1 B). A further indication that Asn117 plays an important role in the interaction with the enolpyruvyl moiety comes from the comparison with the crystal structure of *Bacillus subtilis* MenD (*Bs*MenD, Figure 1 C; PDB ID: 2X7J).[17a] The sequence identity of *Ec*MenD and *Bs*MenD is low (28 %); Pro116 in *Bs*MenD, which defines the conformation of the loop, is located in the position of Asn117 found for *Ec*MenD. Nevertheless, in the position of the lateral chain of *Ec*MenD Asn117, lies the lateral chain of *Bs*MenD Asn431. It is therefore likely that this amide group is a crucial factor for coordination of the enolpyruvyl moiety of **2**.

The previous docking models of *Ec*MenD with **2** predicted the same residues (Ile474, Leu478, Phe475) as interacting with the unsubstituted ring edge; however, the reported orientation of **2** was different.[15,18] Arg107 and Arg33 are known to be important for the binding of **2**:[15,18] mutation of these two moieties to lysine led to a smaller $k_{cat}$ with the analogue 2-succinyl-5-carboxymethoxy-6-hydroxycyclohex-3-ene-1-carboxylate, relative to isochorismate (**2**).[18] As this analogue and **2** differ solely in the enolpyruvyl moiety, it was suggested that Arg107 and Arg33 interact with this moiety.[18] However, as **2** has two carboxylic acid moieties, it is difficult to unequivocally judge which of the carboxylates binds to each Arg residue. Recently, *M. tuberculosis* MenD (*Mtb*MenD) was crystallized with bound **2**.[23] Although *Ec*MenD and *Mtb*MenD share low sequence identity (30 %), some residues that interact with **2** are conserved and are positioned in a similar manner concerning this substrate, namely Ile474, Leu478, Phe475, Arg107, and Gln118. In particular, Arg107 was shown to bind the C1-carboxyl and C6-hydroxy groups, and Gln118 the C6-hydroxy group,[23] as predicted for *Ec*MenD in our model.[24] Regarding the loop that interacts with the enolpyruvyl moiety, in *Mtb*MenD the side chain of Arg282 is involved in a bidentate interaction, while this residue is not conserved in *Ec*MenD.

To produce **8** in vivo selectively, the active site of MenD needed to be modified in such a way that the native substrate **2** is refused. According to the new binding model, residue Arg107 coordinates structural elements that are common to **2** and 2,3-*trans*-CHD (**4**), namely the C1-carboxyl and C6-hydroxy groups. A mutation of this residue is thus expected to affect the activity in the same way for both substrates. To test this hypothesis, Arg107 was mutated to the sterically demanding tyrosine. The production of **7** and **8** was lowered 13-fold in both cases, confirming our assumption (Figure 2).

As **2** and **4** differ exclusively in the enolpyruvyl moiety, the mutations were focused in the region of the active site interacting with this group. Firstly, Asn117 of MenD was predicted to establish a hydrogen bond with the enolpyruvyl moiety of the substrate. To maintain this bond with the C3-hydroxy group of **4**, the alternative residues Gln, Lys, Arg, Thr, and Asp were selected. The variants Asn117Asp, Asn117Gln, and Asn117Thr showed a similar **8**/**7** ratio compared to wild-type MenD (about 2:1), while mutation of Asn117 to Lys and Arg resulted in an increased **8**/**7** ratio to about 8:1 and 6:1, respectively (Figure 2). The proportion of unconsumed **4** increased for most variants, but was not significantly affected for Asn117Gln and Asn117Arg.

Further, the moiety Leu478 was targeted. This residue is located in a hydrophobic pocket, in which Ile474, Leu477, and

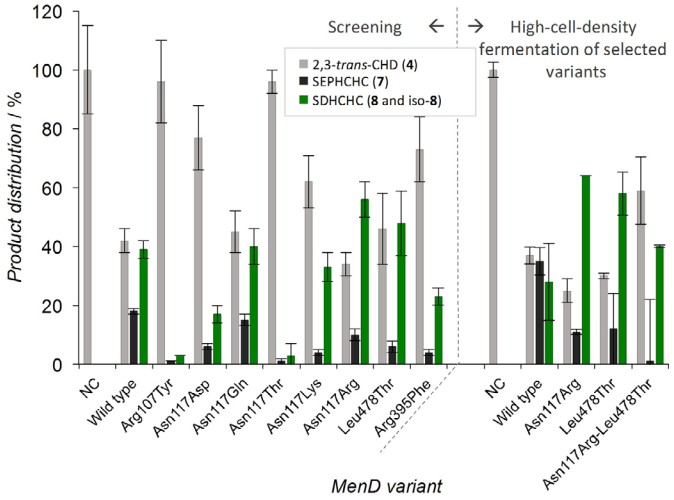


**Figure 2.** Fermentation profile of MenD variants for the conversion of **4**. For each variant, 100% corresponds to the molar sum of unconsumed **4**, and the products **7** and **8**/iso-**8**.

Phe475 form a loop (Figure 1A), the flexibility of which could be important for catalysis. In vitro studies with the variants Leu478Glu and Leu478Lys showed a lower relative enzymatic activity with **4** (43 and 80%, respectively). The variant Leu478Thr, which did not compromise the conversion of **4**, was expressed in *E. coli* and resulted in a higher **8**/**7** ratio (ca. 8:1). To confirm the results of the variant screenings, high-cell-density fermentations were performed for the MenD single variants Asn117Arg and Leu478Thr, as well as for the double variant Asn117Arg–Leu478Thr (Figures 2 and S2). For the single variants, the titer of SDHCHC (**8** and iso-**8**) was in the range of 3.3–5.3 g $L^{-1}$, while for wild-type MenD it was 1.9 g $L^{-1}$. For the double-variant MenD Asn117Arg-Leu478Thr, SDHCHC formation was lower than for the single variants; nevertheless, the **8**/**7** ratio was the highest (>70-fold than that for the wild type).

To further increase the chemical diversity, 2,3-*trans*-CHA (**5**) was investigated as a substrate of MenD. In previous work, the envisaged product 2-succinyl-6-amino-5-hydroxycyclohex-3-ene-1-carboxylate (SAHCHC, **10**; Scheme 1) was not found,[11] possibly due to detection limits. To improve the sensitivity, we established an LC-MS/MS method based on hydrophilic interaction chromatography (HILIC) to investigate the conversion of **5** into **10**. The parent ion $[SAHCHC-H^+]^-$ (*m/z* 255.9) was fragmented and the transition ions (*m/z* 255.9→133.0 and *m/z* 255.9→211.7) were used for the quantification by multiple reaction monitoring scans. The detection limit was thus lowered nearly 1000-fold relative to the previously employed NMR method.[11] A concentration of 0.09 mM **10** (1.5% conversion) was quantified for the MenD-catalyzed enzymatic reaction of **5** with α-ketoglutarate (**6**); hence, (trace) activity with **5** as a substrate has been detected for the first time.

Based on the docking model (Figure 1), we hypothesized that Arg107 is the main impediment for the conversion of **5**. Arg107 lies next to the hydroxy moiety of **2**. The side chain of Arg, when positively charged, might repulse the amino moiety of **5**. The surface potential of the active site of MenD indicates that the substrate channel is dominated by positive charges. Previous studies have shown that the loop composed of Arg107–Ala119 has a strong influence on the conversion of 2,3-*trans*-CHD (**4**; Figure 1);[24] in particular, the amino acid Gln118, that lies at the end of the loop, is crucial for enzyme activity. Thus, mutation of Gln118 of MenD to either Glu, His, or Ala reduced the activity with **4**.[24]

Our variations of Arg107 were therefore designed to minimize the perturbation of this loop. To reduce the steric hindrance and remove the positive charge, Arg107 was exchanged for Glu, Lys, Ile, or Tyr. The variant Glu107 also fulfilled the goal of introducing a negatively charged side chain that could interact with the (putatively positively charged) amine of **5**. The obtained variants were expressed and purified, and assayed in vitro for the conversion of **4** and **5** (Figures 3 and S3). The variants showed up to 23-fold conversion of **5** relative to wild-type MenD; concomitantly, the conversion of **4** was lowered to 5–20% relative activity.

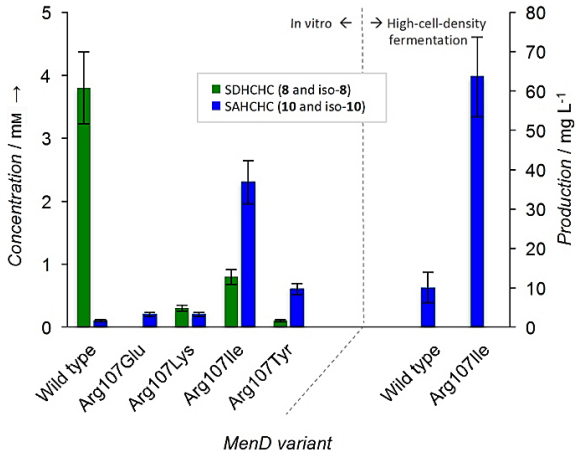


**Figure 3.** In vitro activity of MenD variants with 2,3-*trans*-CHD (**4**) and 2,3-*trans*-CHA (**5**) as the substrates, and SAHCHC (**10** and iso-**10**) production in high-cell-density fermentations.

With the same strategy as used for the in vivo conversion of **4**, a 2,3-*trans*-CHA (**5**) overproducer strain was used for the expression of MenD. Following the approach used by Bongaerts et al.,[9] *E. coli* F68/*pC35-2* was obtained by expressing two genes (*phzD-phzE*) from *Pseudomonas aeruginosa* encoding ADIC synthase, which forms **3** that is hydrolyzed by the isochorismatase PhzD to **5** (Scheme 1).[25] Genes encoding wild-type MenD and MenD Arg107Ile were cloned into this strain, resulting in yields of 10 and 64 mg $L^{-1}$ SAHCHC (**10**), respectively.

Analogously to iso-**8**, iso-SAHCHC (iso-**10**), was purified and its structure elucidated by NMR spectroscopy. In accordance to the data obtained for the regioisomer iso-**8**, a methylene group was identified in the ring. The cross-coupling between the two multiplet signals at 2.30–2.40 and 2.65–2.70 ppm to the C4 carbon (HSQC), proved the presence of two diastereotopic protons. Again, the three-bond coupling between H3

and C1′ (HMBC) provided proof of the occurrence of an α,β-unsaturated carbonyl in iso-**10** (Scheme 1).

The shikimate pathway provides access to primary and secondary metabolites, several of them in use as pharmaceuticals or herbicides.[6] The diversity of metabolites is rooted in the multifunctional reactivity of chorismate (**1**) and isochorismate (**2**), as well as in the diversity and the promiscuity of enzymes acting on these two regioisomers. Several new (natural) enzymatic transformations of **1** or **2** have been recently added to the previously known biosynthetic branches of the shikimate pathway.[4,26] We aimed at inserting "unnatural" new products into the plethora of possible enzymatic products by rational variation of enzymatic activity towards new substrates, themselves produced by metabolic engineering. Starting from an enhanced shikimate pathway and making use of the (natural and tailored) promiscuity of MenD, the highly functionalized compounds SDHCHC (**8**) and SAHCHC (**10**), and the regioisomers iso-**8** and iso-**10**, were made accessible in vitro and in vivo.

The trace activity observed for wild-type MenD with one of the newly accomplished substrates (**5**, 1.5% conversion) suggests that similar traits might exist in nature. Moreover, the rationally designed variation Arg107Lys (position 102 according to the standard numbering scheme of thiamine diphosphate dependent decarboxylases[27]) can be found in enzyme candidates from different bacteria, although at a very low abundance (0.3% of all MenD homologues in contrast to more than 94.0% having Arg102). Based on pairwise sequence identities of those MenD homologues that contain residues other than arginine at standard position 102, a protein sequence network was formed (Figure 4 and the Supporting Information).

One community of this network predominantly contained sequences with Gln and individual sequences with Lys, Ile, or Tyr among other variants (Supporting Information). It is tempting to assume that several variants of MenD homologues with yet unexplored substrate scope already occur in nature, including the mutations at Arg102 assessed in the present study. The possible biochemical peculiarities of the strains encoding these MenD homologues might be a promising starting point for the identification of uncommon MenD-catalyzed reactions. Although **5** might be too stable to represent a general branching point,[29] highly reactive ADIC (**3**) and aminodeoxychorismate might well be. Accordingly, we propose that the chorismate-isochorismate "tree" possesses as yet unexplored branches and its "fruits", the secondary metabolites, are even more diverse than previously suspected.

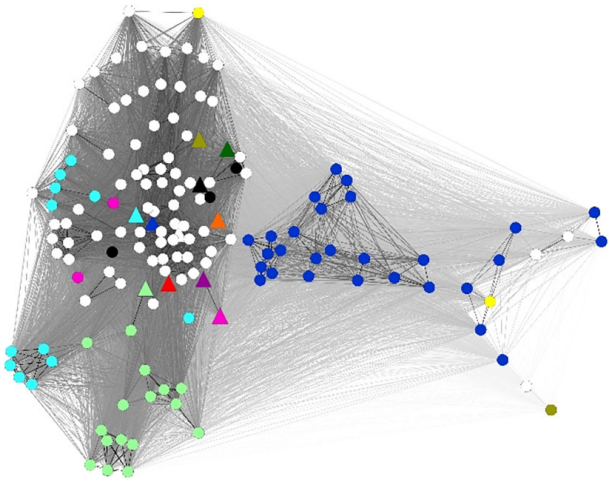

**Figure 4.** Protein sequence network for 150 MenD-homologues without Arg at standard position 102 based on pairwise Needleman–Wunsch alignments.[28] Ala: orange; Gly: yellow; His: cyan; Ile: dark green; Lys: blue; Leu: light pink; Met: black; Asn: light green; Pro: ocher; Gln: white; Val: dark pink; Tyr: red. Eleven sequences for the exemplary alignment are marked as triangles (Supporting Information).

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## Conflict of Interest

*The authors declare no conflict of interest.*

**Keywords:** diversity-oriented synthesis · enzyme catalysis · metabolic engineering · synthetic biology · thiamine diphosphate

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[29] 2,3-*trans*-CHA (**5**) is an intermediate in the biosynthesis of phenazines: W. Blankenfeldt, A. P. Kuzin, T. Skarina, Y. Korniyenko, L. Tong, P. Bayer, P. Janning, L. S. Thomashow, D. V. Mavrodi, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 16431 – 16436, and is proposed to be an intermediate in the biosynthesis of tilivalline and limazepine.

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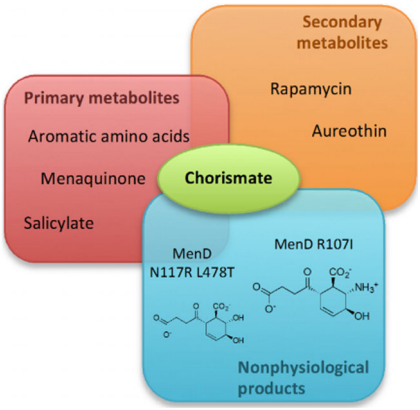
# COMMUNICATIONS

**Unnatural chorismate-derived metabolites**: The route to menaquinone has been modified and the substrate range of the enzyme MenD tailored for the synthesis of nonphysiological compounds. The results demonstrate that a few mutations are sufficient to modify substrate recognition by MenD, hence raising the question of the occurrence of these or similar, but so far unknown, transformations in nature.

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**Alteration of the Route to Menaquinone towards Isochorismate-Derived Metabolites**




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