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Diversity-Oriented Production of Metabolites Derived from Chorismate and Their Use in Organic Synthesis**

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Dedicated to Professor Heinz G. Floss

According to Corey's retrosynthetic approach,[1] the chemical synthesis of individual target compounds requires the use of specific starting synthons in most cases. In contrast, the matrix approach[2] inspired by nature's biosynthetic machinery is based on a diversity-oriented strategy. Indeed, the in vivo synthesis of many natural metabolites is subject to a complex matrix of dependencies and regulations. Thus, natural metabolites may generally be biosynthesized by alternative pathways, starting either from different or from the same compounds. The biosynthesis of a metabolite may require one specific enzymatic transformation or a biotransformation step that is catalyzed by several enzymes acting together in a cascade of reactions. Moreover, the activity of some enzymes may differ from substrate to substrate, some enzymes may simultaneously be involved in several different pathways, and other enzymes may be diversified in posttranslational modification steps.[3] Furthermore, the status of the matrix is generally controlled on the DNA level by regulating the enzyme expression, and on the metabolite level by activating or inhibiting enzyme activities.

The shikimate pathway is one prominent example of a matrix-based biosynthesis, which is essential in plants, bacteria, and fungi,[4] and has been described as a branched metabolic tree for the synthesis of a wide range of (mostly aromatic) compounds. Several enzymes that modify chorismate exhibit structural and mechanistical similarities. The biosynthesis of chorismate and its precursors is subject to complex regulations.[5,6] Some important enzymatic transformations that start from chorismate (**1**)[7] are depicted in Scheme 1. The proteinogenic aromatic amino acids, folates, ubiquinones, menaquinones, enterobactin, and many secondary metabolites are biosynthesized in a few steps starting from chorismate.[5,8]

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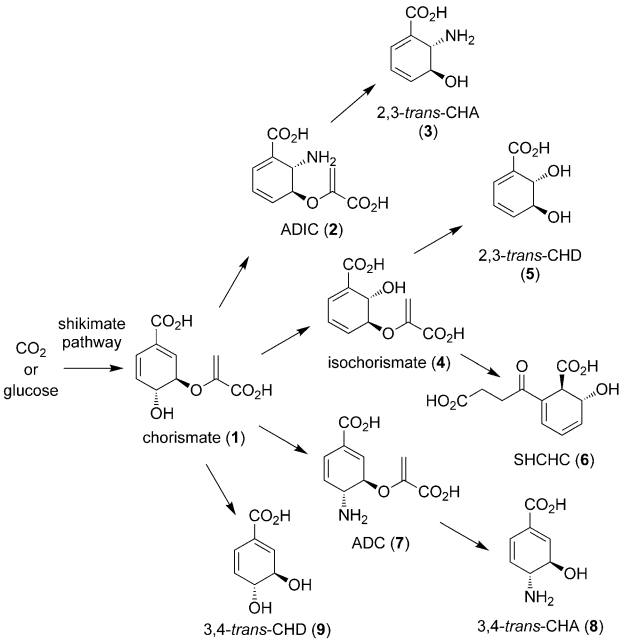
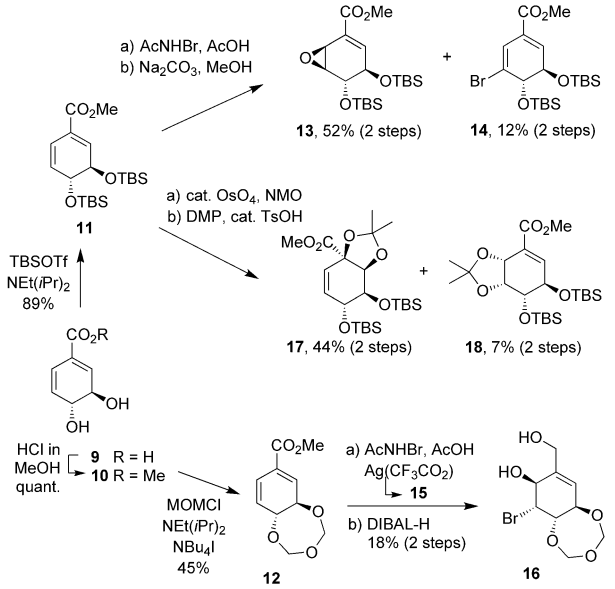


Scheme 1. Recently described metabolites derived from chorismate (**1**). ADIC = 2-amino-2-deoxyisochorismate, ADC = 4-amino-4-deoxychorismate, CHD = cyclohexadienediol carboxylate, CHA = cyclohexadieneaminoalcohol carboxylate, SHCHC = (1*R*,6*R*)-2-succinyl-6-hydroxy-2,4-cyclohexadiene carboxylate.

The aim herein was to use the biosynthetic strategy as a model for producing diverse compounds based on one major biosynthesis pathway. To exemplify this strategy, we chose the shikimate pathway with chorismate as the branching point for the production of a broad array of non-aromatic compounds (Scheme 1). In theory, however, this approach is not restricted to these compounds.

As it has been shown by Leistner and co-workers,[9] strains of *Klebsiella pneumoniae* with deficiencies in the aromatic amino acid pathway excrete (5*S*,6*S*)-5,6-dihydroxycyclohexa-1,3-dienecarboxylic acid (2,3-*trans*-CHD, **5**) and (3*R*,4*R*)-3,4-dihydroxycyclohexa-1,5-dienecarboxylic acid (3,4-*trans*-CHD, **9**) when enzymes catalyzing the conversion of chorismate (**1**) towards these metabolites are overproduced. We have previously described microbial access to **5** and **9** with recombinant *E. coli* strains.[10,11] Improvement in the genetic construction of the relevant strains (deletion of competing metabolic pathways towards phenylalanine and tyrosine, deregulation of precursor flux through the shikimate pathway, enhancement of enzyme activities for the chorismate-converting enzymes EntB and EntC for **5**, enhancement of the activity for EntB, deletion of the *entC* gene for **9**) and improvements of the fermentation process (that is, application of closed-loop control of glucose supply and indirect control for optimum tyrosine and phenylalanine feed during cell growth in 7.5 L or 42 L fed-batch fermentations, respectively) ultimately resulted in a high final concentration of the product of more than 15 $g\,L^{-1}$ for both metabolites together with only low acetate titers. By means of a 300 L fed-batch approach, both compounds were produced on a kilogram scale, and could also be separated by in situ product recovery (ISPR) using reactive extraction.[12] Enantiopure **5** and **9**, as well as the other compounds shown in Scheme 1, are hardly accessible by chemical synthesis. Thus, **5** and **9** became available for subsequent chemical modifications for the synthesis of natural products and pharmaceutically relevant compounds.

The first chemical conversions aimed at the synthesis of structurally related cyclitols. We showed in earlier work that 2,3-*trans*-CHD (**5**) is a beneficial building block for the synthesis of natural product derivatives by stereo- and regioselective epoxidation and dihydroxylation.[13,14] In a similar approach, 3,4-*trans*-CHD (**9**) was regio- and stereoselectively functionalized on each of the two carbon–carbon double bonds. Steric hindrance and induced preferential conformations are the key issues for stereoselectivity. Thus, the hydroxy groups of 3,4-*trans*-CHD methyl ester (**10**) were protected with either sterically demanding *tert*-butyldimetylsilyl (TBS) groups (**11**), or by derivatization to a conformationally rigid [1,3,5]-trioxepine system (**12**; Scheme 2). Subsequent oxidation of **11** with *N*-bromoacetamide took selectively place at the less substituted double bond and resulted in the formation of two isomeric brominated compounds with a ratio of 3:1. Treatment of a methanolic solution of a mixture of both compounds with solid sodium carbonate led to the formation of epoxide **13** and the brominated 3,4-*trans*-CHD derivative **14**. The oxidation of the bicyclic diene **12** with *N*-bromoacetamide in the presence of silver(I) and acetic acid yielded exclusively in one product **15**. The configuration of **15**

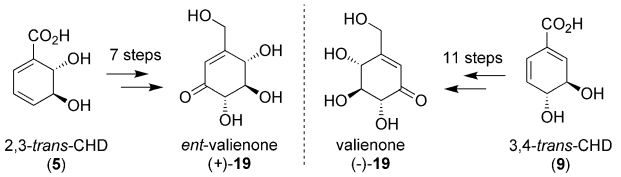


Scheme 2. 3,4-*trans*-CHD (**9**) as a building block in a diversity-oriented synthesis. The molecular structure of **16** was determined by X-ray structure analysis (see the Supporting Information). MOM = methoxymethyl, TBS = tert-butyldimethylsilyl, DMP = 2,2-dimethoxypropane, NMO = 4-methylmorpholine 4-oxide, DIBAL-H = diisobutylaluminum hydride.

was determined by subsequent reduction with DIBAL-H and single-crystal X-ray structure analysis of the resulting diol **16** (see the Supporting Information). The *cis*-selective dihydroxylation of **11** with a mixture of 4-methylmorpholine-4-oxide and catalytic amounts of potassium osmate(VI) occurs predominantly at the more highly substituted double bond. Two regioisomers were obtained in a ratio of 4:1 and 70% yield. Subsequent acid-catalyzed conversion of the mixture of both diols with 2,2-dimethoxypropane (DMP) resulted in the formation of the two acetonides **17** and **18**. The relative stereochemistry of **17** was shown by cleavage of the TBS ethers, which resulted in a compound described by Myers et al.[15] The relative stereochemistry of **18** was elucidated by NOE-NMR experiments after cleavage of the TBS ethers. Surprisingly, and in contrast to our experience with 2,3-*trans*-CHD (**5**), the major products of all reactions examined to date result from an attack *cis* to the adjacent allylic TBS ether of **11** (Scheme 2).

Combined, the microbial access to 2,3-*trans*-CHD (**5**) and 3,4-*trans*-CHD (**9**) enables the efficient synthesis of a variety of different cyclitols and derivatives thereof within a few synthetic steps. The complementary of **5** and **9** was emphasized by the synthesis of both enantiomers of the natural product valienone (**19**) starting from these compounds (Scheme 3).[16]

1,2-Amino alcohols are prevalent in natural products and pharmaceuticals, for example in adrenaline and beta blockers. Furthermore, they serve as chiral auxiliaries and ligands for organic synthesis. Amino acids are another group of compounds that are omnipresent in nature. Besides proteinogenic α-amino acids, which are ubiquitous building blocks in nature, numerous bioactive compounds are amino acids, such as the



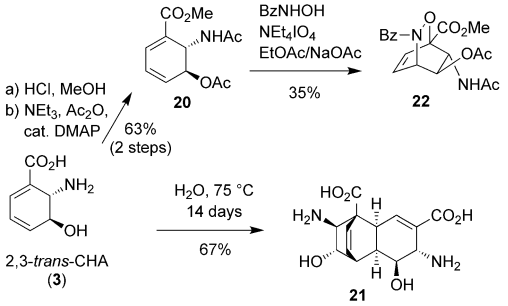
Scheme 3. Synthesis of both enantiomers of valienone (**19**) starting from dienes **5** and **9**.[16]

inhibitory neurotransmitter GABA (γ-aminobutyric acid) and L-DOPA.

Both structural features of a 1,2-amino alcohol and amino acid are combined in the aminocyclitols (5*S*,6*S*)-6-amino-5-hydroxy-cyclohexa-1,5-dienecarboxylic acid (2,3-*trans*-CHA, **3**) and (3*R*,4*R*)-4-amino-3-hydroxy-cyclohexa-1,5-diene carboxylic acid (3,4-*trans*-CHA, **8**). Thus, besides the two diols **5** and **9**, access to these related amino alcohols **3** and **8** expands the range of enantiopure building blocks considerably. Microbial access to **3** has been described previously by McCormick et al. using an uncharacterized mutant of *Streptomyces aureofaciens*.[17] Here, strain improvement using a recombinant *E. coli* strain resulted in the microbial production of **3**, **8**, and 4-amino-4-deoxychorismate (ADC, **7**). Moreover, high product concentration facilitated isolation on a preparative scale.

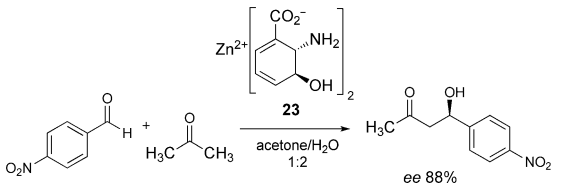
Microbial access to **3** was made possible by expression of the *phzDE* genes from the biosynthesis of phenazines as they occur in *Pseudomonas* strains.[18] Expression of the two genes in *E. coli* cells, which had been improved in the chorismate supply while deleting competing aromatic amino acid pathways, allowed production of up to 12 g L^{-1} of 2,3-*trans*-CHA (**3**) in a 2 L scale, L-tyrosine-limited fed-batch process. By using a 300 L fed-batch approach, **3** was produced on a kilogram scale.[12] Isolation of **3** was performed efficiently by concentration of the cell-free fermentation broth and crystallization from water at 4 °C to obtain **3** in 80 % yield (purity > 95 %). The relative configuration of **3** was verified by single-crystal X-ray structure analysis of the corresponding bisacetyl-protected methylester **20** (see the Supporting Information). Steel et al.[19] recently reported that **3**, synthesized in racemic form, can be oxidized stereoselectively analogous to the oxidation of the diols **5** and **9**. Furthermore, **3** and derivatives thereof have been used in cycloaddition reactions. Although a homomolecular Diels–Alder reaction of **3** requires more drastic conditions compared to **5**, dimerization was achieved by heating a concentrated aqueous solution of **3** for a period of several days. Single-crystal X-ray structure analysis was used to determine the structure of the dimer **21** and putatively also displays the arrangement of the corresponding dimeric compound of 2,3-*trans*-CHD methyl ester (see the Supporting Information). Moreover, we used **5** and **3** as enophiles in a set of different hetero-Diels–Alder reactions. All of the reactions tested proceeded with high stereoselectivity, and the stereochemistry of the reactions such as that leading to **22** was not dependent on the presence or type of protecting groups (Scheme 4).

2,3-*trans*-CHA (**3**) is a new member of the class of cyclic β-amino acids, which are of increasing interest as starting



Scheme 4. 2,3-*trans*-CHA (**3**) as a starting compound for cycloadditions. DMAP = 4-(dimethylamino)pyridine, Bz = benzoyl.

materials for the synthesis of non-natural peptides.[20] Furthermore, **3** can serve as a chiral catalyst for asymmetric synthesis, as is known for α-amino acids such as proline. For example, by using Zn^{2+}-CHA complex **23** as a catalyst in aqueous solution, we converted 4-nitrobenzaldehyde and acetone[21] into the corresponding aldol product with an enantiomeric excess of up to 88 % (Scheme 5).[22] This result



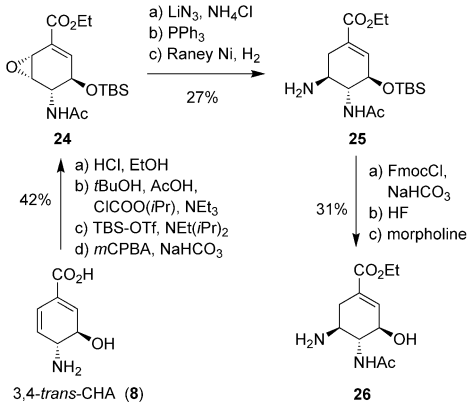
Scheme 5. Asymmetric aldol reaction catalyzed by Zn(2,3-*trans*-CHA)$_2$ **23**.

shows the potential of β-amino acids as chiral catalysts, which is at present underrepresented. In comparison to many other amino acids, **3** allows flexible regio- and stereoselective functionalization, thus facilitating the optimization of putative catalytic properties.

ADC (**7**), an intermediate on the way from chorismate (**1**) to amino acid **8**, was produced to investigate the biosynthesis of **8**. For the production of **7**, the naturally occurring *pabAB* gene fusion from *Corynebacterium glutamicum*[23] was expressed in a recombinant *E. coli* strain with improved flux through the shikimate pathway and with deletions of *pheA* and *tyrA* genes to avoid any drain of chorismate.[24] Compound **7** was produced at up to 7 g L^{-1} in a 2 L scale fed-batch reactor with L-tyrosine limitation; by-products were chorismate and aminodeoxyprephenate. Compound **7** was isolated from the fermentation broth by cation-exchange chromatography.

A culture medium concentration of more than 1.7 g L^{-1} of 3,4-*trans*-CHA (**8**) was obtained by combining *pabAB* gene[25] from *C. glutamicum* and the *phzD* gene from *P. aeruginosa* on one plasmid in a recombinant *E. coli* strain with improved chorismate supply and cultivation of this strain in a 2 L scale L-tyrosine-limited fed-batch process. A by-product was 3,4-*trans*-CHD (**9**), with a final titer of 5.4 g L^{-1}. Compound **8** has not yet been described as a metabolite with biological

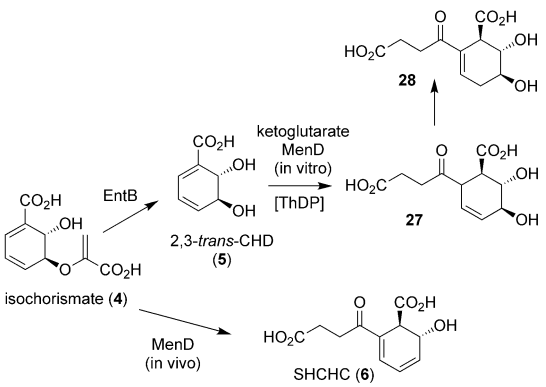
function. In analogy to **7**, this compound was isolated from fermentation broth by cation exchange chromatography. Similar to the β-amino acid **3**, δ-amino acid **8** promises diverse applications as a chiral building block. A prominent example of a structurally related compound is the neuraminidase inhibitor oseltamivir (GS4104),[26] and selective modifications of **8** towards the synthesis of this compound were investigated (Scheme 6).



Scheme 6. Application of 3,4-*trans*-CHA (**8**) as a chiral building block.

Starting from **8**, we synthesized TBS-protected cyclohexene **25** in 7 steps (11 % yield). **25**, which already possesses the fully functionalized skeleton of oseltamivir, was deprotected towards the known amino alcohol **26** (Scheme 6).[27] Apart from the obvious structural relationship of **8** and oseltamivir, natural products such as the monocillinols A and B might be derived and/or synthesized starting from **8**.[28] Moreover, analogous to **3**, the rigid δ-amino acid **8** allows the synthesis of non-natural peptides with new properties through selective modifications of the cyclic system and specific tuning of the conformation.

All of the compounds derived from chorismate (**1**) presented to date combine a cyclohexadiene system with stereogenic centers of secondary alcohols and amines, respectively. By conversion of isochorismate (**4**) into (1*R*,6*R*)-2-(3-carboxypropanoyl)-6-hydroxycyclohexa-2,4-dienecarboxylic acid (SHCHC, **6**), which is a metabolite of the menaquinone pathway,[29] the carbon skeleton is expanded by the addition of a succinyl residue, and a chiral tertiary carbon center is generated selectively. Microbial access to **6** was facilitated by expression of the genes *entC* and *menD*[30] from *E. coli* in a recombinant *E. coli* strain with deficiencies in the biosyntheses of the aromatic amino acids phenylalanine and tyrosine and a lack of MenC activity. Compound **6** was produced with product titers of up to 8.6 $g\,L^{-1}$ in a 2 L scale fed-batch reactor with L-tyrosine limitation, and isolated by anion-exchange chromatography; by-products were chorismate (**1**) and isochorismate (**4**).[31] In vitro experiments starting from 2,3-*trans*-CHD (**5**), 2-ketoglutarate, and MenD resulted in the formation of **27**, which upon isolation tautomerized towards the non-natural new chiral building block **28** (Scheme 7).[32]



Scheme 7. Microbial synthesis of natural SHCHC (**6**) and enzymatic in vitro synthesis of **28**, a novel non-natural chiral building block. ThDP = thiamine diphosphate.

Microbial access to all of the compounds depicted in Scheme 1 (except ADIC (**2**))[18,33] was realized, and *trans*-CHD **5** and **9** as well as 2,3-*trans*-CHA (**3**) were produced on a kilogram scale. Interestingly, the activity of PhzD towards ADC (**7**) enables the bioproduction of **8** and is significantly higher ($K_M = (1.5 \pm 0.3)$ mmol L^{-1}, $k_{cat} = (4.1 \pm 0.12)$ s^{-1}, $k_{cat}/K_M = 2.7 \times 10^3$ L mol^{-1} s^{-1})[24b] than found by Parsons et al. ($K_M = (0.59 \pm 0.14)$ mmol L^{-1}, $k_{cat} = (0.02 \pm 0.002)$ s^{-1}, $k_{cat}/K_M = (34 \pm 24)$ L mol^{-1} s^{-1}).[34] One reason for this discrepancy might be the influence of the different buffers used for the measurements of the enzyme kinetics.[35] In comparison, EntB[36] shows ADC hydrolase activity similar to PhzD ($K_M = (1.4 \pm 0.3)$ mmol L^{-1}, $k_{cat} = (2 \pm 0.05)$ s^{-1}, $k_{cat}/K_M = 1.8 \times 10^3$ L mol^{-1} s^{-1}), but a higher chorismatase activity ($K_M = (1.1 \pm 0.15)$ mmol L^{-1}, $k_{cat} = (56 \pm 3)$ s^{-1}, $k_{cat}/K_M = 40 \times 10^3$ L mol^{-1} s^{-1}), making EntB less appropriate for the conversion of ADC (**7**) into 3,4-*trans*-CHA (**8**). The bioproduction of 3,4-*trans*-CHA (**8**) and SHCHC (**6**) has not yet been optimized with respect to bioreaction engineering.

The versatility of these bioproducts has been demonstrated in natural product syntheses and their value as enantiopure chiral building blocks has been shown. The synthesis of either enantiomer of valienone starting from **5** and **9**, respectively, has been shown exemplarily. Complex chiral structures have been generated selectively by using cycloadditions. Their application as chiral catalysts, as starting materials for chiral ligands, or their use as conformationally rigid amino acids in peptide chemistry are just a few examples of the variety of possibilities.

As the fermentation procedures can be scaled up easily, production of these metabolites can be achieved on a technical scale. The bioproduction of **3**, **5**, and **9** on a 300 L scale yielded more than 15 % product with respect to the glucose consumed during the fermentation process. Apart from their use as fine chemicals, all these compounds promise easy aromatization directly from fermentation broth, as has been shown for dehydroshikimate and dehydroquinate,[37] which would demonstrate their potential as platform chemicals accessible from renewable sources.

The application of ^{13}C- or ^{15}N-labeled substrates results in the formation of specifically labeled metabolites (results not

shown), which are valuable in elucidating as yet unexplored branches derived from the shikimate/chorismate pathway.[38] The microbial production of uncommon structures derived from chorismate (such as echinosporin)[39] becomes feasible as demonstrated by the thiamine diphosphate (ThDP)-dependent MenD-catalyzed C–C bond formation resulting in the production of **6**. Given the multipurpose activity and broad substrate range of ThDP-dependent enzymes,[40] it is justified to suppose that MenD variants or other ThDP-dependent enzymes will accept chorismate, isochorismate, and derivatives as a substrate. This will result in the formation of natural and novel non-natural metabolites such as **27**.

We have demonstrated that single pathways or parts of a biosynthetic matrix can be successfully amplified or suppressed. In this way, the modified matrix becomes an efficient tool for the directed biosynthesis of a valuable single metabolite. Along with the possibility of using the metabolite produced from the biomimetic approach as a renewable resource in diversity-oriented synthesis,[41] the metabolites have another intrinsic advantage: products derived from natural products are privileged structures with regard to biological activity.[42] This method thus enables a smooth development and seamless scale-up, which is desirable for new pharmaceutical approaches.

Experimental Section

Genes of interest (*entB*, *entC*, *phzDE*, *pabAB*) were amplified and cloned by standard PCR and cloning techniques[43] using suitable plasmid cloning and expression vectors (for example pJF119EH)[44] for expression in recombinant *E. coli* K-12 host strains, which had been improved for chorismate supply by enhancing genes of the general aromatic amino acid pathway and by deleting competing pathways for chorismate. All amplified genes were checked for sequence identity. Competing metabolic pathways were eliminated by disruption or deletion of the cognate genes (*pheA*, *tyrA*, *entC*) using standard procedures.[45] Genes *phzDE* from the phenazine biosynthetic pathway[34,46] were amplified from *Pseudomonas aeruginosa*, and the gene *pabAB* encoding ADC synthase was cloned from *Corynebacterium glutamicum* using genomic data of this organism.[23] Details of cloning and strain constructions will be presented elsewhere. *entB* or *entB/C* genes were combined with *aroF*, *aroB*, and *aroL* genes from *E. coli* K-12 on plasmid pJF119EH.

Recombinant *E. coli* strains were incubated in 7.5, 42, and 300 L reactors at 37 °C with a starting aeration of 0.5 volume per volume per minute (vvm). Induction was at an optical density of 6–8 at 620 nm with 0.1 mM IPTG (final concentration). Incubation was carried on for approximately 50 h.

CCDC 641767 (**16**), 641768 (**20**), and 641769 (**21**) contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Abbreviations: EntB = isochorismatase from *E. coli*, EntC = isochorismate synthase from *E. coli*, PhzD = isochorismatase from *P. aeruginosa*, PhzE = 2-amino-2-deoxyisochorismate synthase from *P. aeruginosa*, PabAB = 4-amino-4-deoxychorismate synthase from *C. glutamicum*, MenD = 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate synthase, ADIC = 2-amino-2-deoxyisochorismate, ADC = 4-amino-4-deoxychorismate, CHD = cyclohexadienediol carboxylate, CHA = cyclohexadieneaminoalcohol carboxylate, SHCHC = (1*R*,6*R*)-2-succinyl-6-hydroxy-2,4-cyclohexadiene carboxylate, IPTG = isopropyl thio-ß-galactopyranoside.

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