Easy Access to (*R*,*R*)-3,4-Dihydroxy-3,4-dihydrobenzoic Acid with Engineered Strains of *Escherichia coli*

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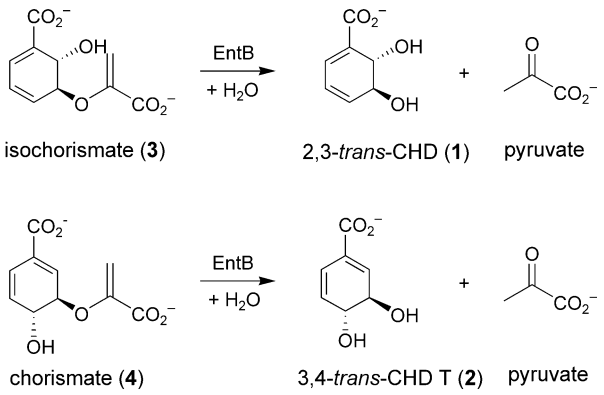
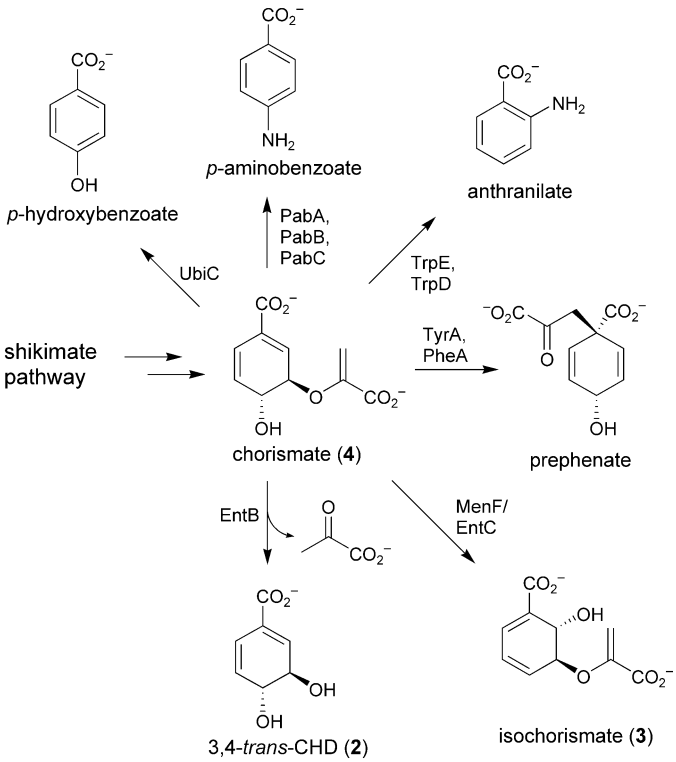
We recently described a preparative route to (*S*,*S*)-2,3-dihydroxy-2,3-dihydrobenzoic acid (2,3-*trans*-CHD [CHD: cyclohexadiene-diol], **1**),[1] that makes use of genetically engineered cells of *Escherichia coli*, based on work previously described by Leistner et al.[2] The potential of 2,3-*trans*-CHD (**1**) as a starting material in natural product chemistry has been demonstrated by the syntheses of *iso*-crotepoxide and *ent*-senepoxide,[3] and the diversity-oriented synthesis of new (amino)carbasugar building blocks.[4] (*R*,*R*)-*trans*-3,4-Dihydroxy-3,4-dihydrobenzoic acid (3,4-*trans*-CHD, **2**), the regioisomer of **1**, promises a variety of synthetic applications similar to those of **1** or to the corresponding *cis*-CHD.[5] In contrast to **1**, which is the metabolic precursor in the biosynthesis of the iron chelator enterobactin, compound **2** has not been found to have a biological function in *E. coli*.[6] Nevertheless, in the presence of isochorismatase (encoded by *entB*), whose physiological function is the hydrolysis of isochorismate (**3**) to form **1**, 3,4-*trans*-CHD (**2**) can be produced from chorismate (**4**) in vitro[7] as well as in vivo with strains of *Klebsiella pneumoniae*[2] (Scheme 1).

We herein report an efficient approach for the production of 3,4-*trans*-CHD (**2**) by redirecting the post-chorismate pathways of aromatic amino acid biosynthesis in *E. coli*. Different approaches for the generation of microbial producers of **2** were examined. Our first approach was analogous to the strategy we used for the production of **1**.[1] Only the pathway for the biosynthesis of enterobactin and menaquinones were blocked, which left the metabolic pathways for aromatic amino acid and folate production intact (Scheme 2, Strategy 1, and Table 1). For this approach, *E. coli* strain PBB8 , which has deficiencies in *entC*/*menF* was transformed with plasmid pDF1, which contains *entB*. Wild-type *E. coli* strain W3110 and *E. coli* strain DH5α were also transformed and used as reference strains (Table 2).



Scheme 1. *Substrates and products of* E. coli *isochorismatase (EntB)-catalyzed transformations.*



Scheme 2. *The strategy for the production of* ***2*** *is based on a higher in vivo activity of isochorismatase (EntB) with* ***4*** *as substrate than that of other enzymes involved in the degradation of* ***4****. Strategy 1:* menF/entC *deleted,* entB *overexpressed. Strategy 2:* pabA, pabB, trpE, tyrA, pheA *deleted,* entB *overexpressed. The enzymes involved are (data from literature, see Table 1): chorismate lyase (UbiC),* p*-aminobenzoate synthase (PabAB), 4-amino-4-deoxychorismate lyase (PabC), anthranilate synthase (TrpED), prephenate dehydrogenase (TyrA), prephenate synthase (PheA), isochorismate synthase (MenF/EntC).*

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Table 1. *Enzymes involved in the degradation of* ***4****.*

Enzyme	K_m [μM][a]	k_{cat} [min^{-1}][b]	k_{cat}/K_m [μM^{-1} min^{-1}]	Reference
UbiC	29	102	3.5	[8]
PabAB	4[c]	30	7.5	[9, 10]
TrpED	4[d]	558[d]	140	[11]
TyrA	45	1620	36	[12]
MenF/EntC	14[e]	173[e]	12.4	[13]
EntB	≥37000[f]	≥1300[f]	≥0.03[d]	[14]

[a] K_m, Michaelis constant. [b] k_{cat}, catalytic rate consant. [c] Determined for the glutamine-dependent reaction of the PabA–PabB complex. [d] Measured in 50 mM $(NH_4)_2SO_4$. [e] Measured in 10 mM $MgCl_2$. [f] Extrapolated to an infinite concentration of **4**.

Table 2. *E. coli K-12 parental strains.*

Strain	Genotype	Reference
DH5α	F$^-$ *endA1 hsdR17*($r_k^- m_k^+$) *recA1 supE44 thi-1* Δ(*lacZYA-argF*)*U169* Φ80*lacZ*ΔM15	[20]
W3110	F$^-$ λ$^-$ IN(*rrnD-rrnE*) prototroph	[21]
BN117	*pabA1 pabB*:Kn *pheA1 tyrA4 trpR*(Tn*10*) Δ*trpEA2 his4 proA2 argE3 rpsL704*	[22]
PBB8	F$^-$ *laclq* Δ(*lon*) *hflA150:Tn10* Δ(*argF-lac*)*169 proA*$^+$ *araD139 rpsL* λ$^-$ *entC*$^-$ *menF*$^-$	[23]

The resulting *E. coli* strains PBB8/pDF1, W3110/pDF1, and DH5α/pDF1 could be grown in mineral salts medium with glucose as the sole carbon source, which has been used as a growth medium for other transformants.[1] Expression of the gene product, EntB, was verified by SDS-PAGE and at least a 160-fold increase of activity for EntB as compared to that found before transformation was observed for all strains by using an enzymatic assay.[4]

However, formation of **2** in the cultivation medium could not be achieved with strain PBB8/pDF1, nor in the reference strains. This problem can be attributed to the meagre k_{cat}/K_m value of EntB with chorismate (**4**) as a substrate; this value cannot compete with the k_{cat}/K_m values of UbiC, PabAB, TrpED, and chorismate mutase, together with prephenate dehydrogenase (TyrA) or prephenate dehydratase (PheA), which have naturally evolved to use **4** as a substrate (Scheme 2, Table 1).

Next, the pathways towards aromatic amino acid and folate production were blocked, in order to eliminate the metabolic degradation and aromatization reaction of **4** (Scheme 2, Strategy 2). *E. coli* strain BN117, which has deletions of genes in the pathways towards the production of *p*-aminobenzoate, anthranilate, and prephenate (*pabA*$^-$, *pabB*$^-$, *trpE*$^-$, *pheA*$^-$, *tyrA*$^-$) was transformed with plasmid pDF1. The resulting strain BN117/pDF1 showed production of the active enzyme EntB at a level similar to that in PBB8/pDF1 and W3110/pDF1.[4] In contrast to PBB8, however, strain BN117 did not grow in mineral salts medium, even when supplied with all the substances that this strain needs for growth (tryptophan, tyrosine, phenylalanine, proline, arginine, histidine, *p*-aminobenzoate).[15]

Further attempts to grow the strain in defined media were unsuccessful. Therefore, cells of BN117/pDF1 were first grown in a complex medium and then resuspended in the mineral salts medium (without supplementation), with glucose as the carbon source. This procedure resulted in excretion of **2** at a rate of 19 mg h^{-1} (g dry cell mass)$^{-1}$ (Table 3). The production of **2** was observed over a time period of 36 h (average formation rate of 10 mg h^{-1} (g dry cell mass)$^{-1}$) and resulted in a final accumulation of 790 mg L^{-1} **2**.

Table 3. *Maximal product formation rate (3 h after induction) and final concentration (after 36 h) of* ***2****. No excretion of* ***1****,* ***3****, or* ***4*** *was observed.*

Strains in use	Rate (3,4-*trans*-CHD (**2**)) [mg h^{-1} (g dry cell mass)$^{-1}$]	Conc. (**2**) [mg L^{-1} (mM)]
DH5α/pDF1, W3110/pDF1	≤0.03	≤1
PBB8/pDF1	≤0.03	≤1
BN117/pDF1	19	790 (5.1)

No metabolic byproducts from the shikimate/chorismate pathway were found in the cultivation medium by ^{1}H NMR spectroscopy or HPLC analysis. Compound **2** was purified on an analytical scale by using preparative HPLC to give **2** in a highly pure form as a yellow solid. The analytical data for this product were identical to those for authentic racemic **2** prepared by a chemical route.[4b, 16] Ion-exchange chromatography was used for isolation and purification of **2** on a preparative scale. From 200 mL cultivation medium (790 mg L^{-1}, 5.1 mM **2**), 110 mg (0.7 mmol) **2** were obtained, which corresponds to 69% yield for the isolation. The chemical purity of the product was determined to be greater than 90% (^{1}H NMR spectroscopy and HPLC analyses). In contrast to chorismate (**4**),[17] **2** is stable over several days at room temperature, both in aqueous solution at a neutral pH value and as lyophilizate.

Compared to chemical synthesis starting from *cis*-CHD[18] and isolation from *Klebsiella* strains,[2] the microbial production of **2** described herein has significant advantages: The isolated product **2** is free of metabolic byproducts, which makes tedious separation processes obsolete. Additionally, the product concentration of **2** described herein (790 mg L^{-1}, 5.1 mM), and the separation of the growth phase and production phase allow high product purity. Finally, *E. coli* strain BN117/pDF1 is non-pathogenic and classified as an S1 organism according to German recombinant DNA regulations.

We are currently using the microbially produced 3,4-*trans*-CHD **2** to evaluate the potential of this chiral substance as a starting material in the synthesis of new carbohydrate mimetics. With compounds **2** and **1** to hand, the synthesis of diverse products stereochemically complementary to those obtained by the chemistry based on *cis*-CHD is enabled in principle.

Experimental Section

The gene *entB* from *E. coli* K-12 wild-type strain W3110 was amplified by the polymerase chain reaction and inserted into plasmid pJF119EH1 as described previously.[1] The resulting plasmid pDF1 was transformed into strains DH5α, W3110, BN117, and PBB8 by using the calcium chloride method.[19]

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Cultivation of BN117/pDF1 to obtain **2** was carried out in mineral salts medium (100 mL)[1] in 1-L shaking flasks. The pH value was adjusted to 7 with 1 N NaOH. Cells from a preculture in Luria – Bertani medium[19] (100 mL, containing 100 mg L^{-1} ampicillin, 100 μM isopropyl β-D-thiogalactopyranoside (IPTG)) were harvested at an optical density of 2 ($\lambda = 600$ nm) by centrifugation (5000 g, 5 °C, 5 min) and were resuspended in mineral salts medium[1] (100 mL, containing 100 mg L^{-1} ampicillin, 100 μM IPTG, and 10 g L^{-1} glucose) and incubated at 37 °C and 150 rpm. Product formation was monitored over a time period of 36 h by analyzing the cell-free supernatant with HPLC by using a method described elsewhere.[4] Maximal product formation was observed 3 h after induction.

Product isolation and characterization: To isolate **2**, cells were removed by centrifugation (5000 rpm, 5 °C, 10 min). For characterization, cell-free cultivation supernatant (100 mL, 790 mg L^{-1} (5.1 mM) **2**) was adjusted to pH 7 and all volatile substances were removed in vacuo. Chromatographic separation was carried out on a preparative HPLC system by using an RP-18 column (Kromasil 10-C18, 250 × 10 mm, CS-Chromatographie Services GmbH, Langerwehe) and applying a linear gradient of aqueous 0.1% trifluoroacetic acid to methanol/aqueous 0.1% trifluoroacetic acid (1:1) at a flow rate of 5 mL min^{-1}. Elution was monitored at a wavelength of 278 nm. From 500 μL concentrate (20 g L^{-1} (128 mM) **2**), 10 mg (64 μmol) **2** were isolated from the fractions collected at 20 – 23 min and obtained as a yellow solid after lyophilization (quantitative yield).

Preparative isolation of 2: Cell-free cultivation supernatant (200 mL, pH 7, 790 mg L^{-1} (5.1 mM) **2**) was concentrated in vacuo and the remaining viscous oil was extracted three times with methanol/ethyl acetate (200 mL, 1:1) over a time period of 14 h for each extraction. All nonsoluble ingredients in the combined organic phases were removed by filtration and volatile substances were evaporated in vacuo to give crude product (630 mg), which was dissolved in water (20 mL) and applied to an anion-exchange chromatography column. DOWEX 1 × 8 resin (20 g, Cl^- form, 5-cm package height) was equilibrated with K_2HPO_4 solution (50 mM, 8.7 g L^{-1}, pH 8, 9.5 mS cm^{-1}) before the crude product solution was passed through the resin. Elution was carried out without a previous wash cycle, with formic acid (50 mM, 2.3 g L^{-1}, pH 2.3, 1.2 mS cm^{-1}) at a flow rate of 16 mL min^{-1}. Compound **2** was eluted after 20 min at pH 2.3. After removal of all volatile substances in vacuo, **2** (110 mg, 0.7 mmol) was obtained as a yellow solid. This corresponds to a 69% yield for the isolation process. The purity of **2** was determined to be more than 90% (^{1}H NMR spectroscopy, HPLC analysis).

Physical data of 2: $[\alpha]_D^{20}$[2] = −1.6 ($c = 0.1$ in H_2O); $[\alpha]_D^{20}$ (found) = −1.6 ($c = 0.1$ in ethanol); ^{1}H NMR (300 MHz, [D_4]MeOH, 23 °C): 4.47 (dt, $J = 10.3$, 2.3 Hz, 1 H; C*H*OH), 4.55 (dd, $J = 10.3$, 3.4 Hz, 1 H; C*H*OH), 6.03 (dd, $J = 9.9$, 3.1 Hz, 1 H, C=C*H*), 6.28 (d, $J = 9.9$ Hz, 1 H, C=C*H*), 6.60 (s,1 H, C=C*H*) ppm; ^{13}C NMR (75 MHz, [D_4]MeOH, 23 °C): $\delta = 74.2$ (CH), 75.1 (CH), 122.7 (CH), 129.4 (C_{quat}), 133.4 (CH), 141.4 (CH), 168.3 (C=O) ppm; IR(KBr): $\tilde{\nu} = 3385$ (O–H), 1661 (C=O), 1645 (C=C), 1600 (C=C), 1406, 1260, 1078, 1016 cm^{-1}.

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