Biosynthesis of Two Hydroxybenzoic Acid-Amine Conjugates in Engineered *Escherichia coli*

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Two hydroxybenzoyl amines, 4-hydroxybenzoyl tyramine (4-HBT) and *N*-2-hydroxybenzoyl tryptamine (2-HBT), were synthesized using *Escherichia coli*. While 4-HBT was reported to demonstrate anti-atherosclerotic activity, 2-HBT showed anticonvulsant and antinociceptive activities. We introduced genes chorismate pyruvate-lyase (*ubiC*), tyrosine decarboxylase (*TyDC*), isochorismate synthase (*entC*), isochorismate pyruvate lyase (*pchB*), and tryptophan decarboxylase (*TDC*) for each substrate, 4-hydroxybenzoic acid (4-HBA), tyramine, 2-hydroxybenzoic acid (2-HBA), and tryptamine, respectively, in *E. coli*. Genes for CoA ligase (*hbad*) and amide formation (*CaSHT* and *OsHCT*) were also introduced to form hydroxybenzoic acid and amine conjugates. In addition, we engineered *E. coli* to provide increased substrates. These approaches led to the yield of 259.3 mg/l 4-HBT and 227.2 mg/l 2-HBT and could be applied to synthesize diverse bioactive hydroxybenzoyl amine conjugates.

Keywords: Hydroxybenzoic acid, *N*-2-hydroxybenzoyl tryptamine, 4-hydroxybenzoyl tyramine, metabolic engineering

Introduction

Hydroxybenzoic acids are common chemicals found in plants. 2-HBA (2-hydroxybenzoic acid) serves as a signal molecule in plants [1] and 4-HBA (4-hydroxybenzoic acid) is a precursor for the synthesis of ubiquinol-6 in eukaryotes [2, 3]. These two compounds form amines with either tyramine or tryptamine; 4-Hydroxybenzoyl tyramine (4-HBT), found in *Houttuynia cordata* [4], has been reported to show anti-atherosclerotic activity [4]. *N*-2-hydroxybenzoyl tryptamine (2-HBT) has been studied as a benzoyl tryptamine analogue owing to its anticonvulsant [5, 6] and antinociceptive activities [7]. 2-HBT has been shown to relieve acute and chronic pain and inflammation [8].

Recently, biological synthesis of various chemicals, using *Escherichia coli*, has become prevalent. The biological pathway for the synthesis of target compounds is newly introduced into *E. coli* and engineered to increase the supply of substrates in *E. coli*. Precursors of 4-HBT and 2-HBT are available in *E. coli*; 4-HBA and 2-HBA are synthesized from chorismate [9, 10]. Tyramine and

tryptamine are from tyrosine and tryptophan, respectively [11, 12]. Various BAHD (benzyl alcohol *O*-acetyltransferase (BEAT), anthocyanin *O*-hydroxycinnamoyl transferase (AHCT), anthranilate *N*-hydroxycinnamoyl/benzoyl transferase (HCBT), and deacetyl vindoline 4-*O*-acetyltransferase (DAT)) acyltransferase families are known for amide formation between hydroxybenzoic acid and tyramine or tryptamine [13]. By expressing these genes in *E. coli*, amide formation between phenolic acid and various amines including tyramine, dopamine, tryptamine, and serotonin was conducted [14–16]. Combination of amine synthesis and hydroxybenzoic acid pathways in *E. coli* is considered to enable the synthesis of a new amide. Here, we report the synthesis of two new amides in *E. coli*, namely 4-HBT and 2-HBT. The route of synthesis for two hydroxybenzoic acids (4-HBA and 2-HBA) was introduced into *E. coli*. *UbiC* gene was overexpressed to provide a substrate for 4-HBT, and two genes, *entC* and *pchB*, were overexpressed to synthesize 2-HBA (Fig. 1). In addition, we engineered the shikimate pathway of *E. coli* to provide more substrates for the synthesis of 4-HBT and 2-HBT.

Fig. 1. Schematic pathway for the synthesis of 4-hydroxybenzoyl tyramine and *N*-2-hydroxybenzoyl tryptamine in *E. coli*.

PpsA, phosphoenolpyruvate synthase; TktA, transketolase; AroG, phospho-2-dehydro-3-deoxyheptonate aldolase; AroL, shikimate kinase; EntC, isochorismate synthase; PchB, isochorismate pyruvate-lyase; Tdc, tryptophan decarboxylase; Hbad, hydroxybenzoate coenzyme A ligase; OsHCT, hydroxycinnamoyl transferase from *O. sativa*; TyDC, tyrosine decarboxylase; TyrA, prephenate dehydrogenase; TyrB, aromatic amino acid aminotransferase; UbiC, chorismate pyruvate lyase; CaSHT, serotonin *N*-hydroxycinnamoyl transferase from *Capiscum annuum*.

Materials and Methods

Constructs

PsTyDC and *CaSHT* were cloned previously [15]. *PsTyDC* was subcloned into BamHI/HindIII site of pETDuet-1 vector. The *ubiC* gene (CAA47181), encoding chorismate pyruvate-lyase from *E. coli*, was cloned by polymerase chain reaction (PCR) using primers 5'-aacatATGTCACACCCCGCGT-3' and 5'-aaggtaccTTAGTAC AACGGTGACGCC-3' (NdeI and KpnI sites are underlined), and then subcloned into NdeI/KpnI site of the pETDuet-1 containing *PsTyDC*. *hbad* gene (hydroxybenzoic acid-CoA ligase; U02033) was amplified with genomic DNA of *Rhodopseudomonas palustris* with primers 5'-aaggtaccaGGATCCAATGCCGCTACGCGACTACA-3' and 5'-aagcgccgcTCATCGTCCGTTGCCGG-3' (BamHI and NotI sites are underlined) and then subcloned into BamHI/NotI site of pCDFDuet-1. *CaSHT* was subcloned into the BglIII/KpnI site of pCDFDuet-1 harboring *hbad* (pC-hbad-*CaSHT*). BamHI site of *tyrA*^f was deleted without any amino acid change and the resulting gene was subcloned into NdeI/KpnI of pACYCDuet-1 (pA-*tyrA*^f). *aroG*^f-*ppsA*-*tktA* and *aroL*-*aroG*^f-*ppsA*-*tktA*, each containing a ribosome binding site (RBS), were subcloned into BamHI/NotI site of pA-*tyrA*^f.

OsHCT (Os11g42370) was cloned using RT-PCR with primers, 5'-aagaattcaATGGAGATCACGAGCAGCG-3' and 5'-aagcgccgc

TTAAAGATGAGAGGGGATAGCATG-3' and then subcloned into EcoRI/NotI site of pETDuet-1. *BaTDC* was subcloned into EcoRV/XhoI site of pETDuet-1 carrying *OsHCT*. Two genes, *entC* from *E. coli* and *pchB* from *Pseudomonas fluorescens*, were cloned into NdeI/KpnI site of pCDFDuet-1 carrying *hbad*.

The promoter of *trp* operon in *E. coli* was replaced with T7 to create the strain BT7P (Table 1). This was done by a two-step PCR: the first PCR was performed with FRT-PGK-gb2-neo-FRT DNA as a template, using 5'-tggtatatCTCCTTattaaagtttaacaaaattaCTA TAGTGAGTCGTATTAaatacagactcactatagggtc-3' (underlined capital letters show ribosome binding site (RBS), capital letters represent T7 promoter sequence, and underlined sequences are from FRT-pgk-gb2-neo-FRT cassette) as the forward primer and 5'-TGGATAATGTTTTTGCGCCGACATCATAACGGTCTGGCA AATATCTCGattaaccctcactaaaggcg-3' (underlined capital letters are from *trp* operon and lower case letters are from FRT-pgk-gb2-neo-FRT cassette) as the reverse primer. The second PCR was performed with the template generated from the first PCR, using 5'-TAAGCGCCTTCGCAGGTTAGCTGTTTCGAGAGTCGGTTTTTG TGTTCATtggtatatctcctattaaa-3' (capital letters are from *trp* operon promoter region and underlined sequences are part of the first PCR product) as the forward primer and reverse primer from the 1st PCR. The second PCR product was used to replace the native *trp* promoter of *E. coli* with a T7 promoter using the Quick

Table 1. Plasmids and strains used in the present study.

Plasmids or <i>E. coli</i> strains	Relevant properties or genetic marker	Source or reference
Plasmids		
pACYCDuet-1	P15A ori, Cm ^r	Novagen
pCDFDuet-1	CloDE13 ori, Str ^r	Novagen
pETDuet-1	f1 ori, Amp ^r	Novagen
pC-hbad-CaSHT	pCDFDuet-1 + <i>hbad</i> from <i>Rhodospseudomonas palustris</i> and <i>CaSHT</i> from <i>Capiscum annuum</i>	This study
pE-PsTyDC-ubiC	pETDuet-1 + <i>PsTyDC</i> from <i>Papaver somniferum</i> and <i>ubiC</i> from <i>Escherichia coli</i>	This study
pA-aroG ^f -tyrA ^f	pACYCDuet-1 + <i>aroG^f</i> and <i>tyrA^f</i> from <i>E. coli</i>	This study
pA-aroG ^f -ppsA-tktA-tyrA ^f	pACYCDuet-1 + <i>aroG^f</i> , <i>ppsA</i> , <i>tktA</i> , and <i>tyrA^f</i> from <i>E. coli</i>	This study
pA-aroL-aroG ^f -ppsA-tktA-tyrA ^f	pACYCDuet-1 + <i>aroL</i> , <i>aroG^f</i> , <i>ppsA</i> , <i>tktA</i> , and <i>tyrA^f</i> from <i>E. coli</i>	This study
pC-hbad	pCDFDuet-1 + <i>hbad</i> from <i>R. palustris</i>	This study
pC-hbad-entC-pchB	pCDFDuet-1 + <i>hbad</i> from <i>R. palustris</i> and <i>entC</i> from <i>E. coli</i> and <i>pchB</i> from <i>Pseudomonas fluorescens</i>	This study
pE-OsHCT	pETDuet-1 + <i>HCT</i> from <i>Oryza sativa</i>	This study
pE-OsHCT-BaTDC	pETDuet-1 + <i>HCT</i> from <i>O. sativa</i> and <i>TDC</i> from <i>Bacillus atrophaeus</i>	This study
pA-aroL-aroG ^f -ppsA-tktA	pACYCDuet-1 + <i>aroL</i> , <i>aroG^f</i> , <i>ppsA</i> , and <i>tktA</i> from <i>E. coli</i>	This study
Strains		
BL21 (DE3)	F ⁺ <i>ompT hsdS_B(r_B⁻ m_B⁻) gal dcm lon</i> (DE3)	Novagen
B-tyrR	BL21(DE3) Δ <i>tyrR</i>	Kim et al. (2013)
B-tyrR/pheA	BL21(DE3) Δ <i>tyrR</i> / Δ <i>pheA</i>	Kim et al. (2013)
B-trpEG/pheA	BL21(DE3) Δ <i>trpEG</i> / Δ <i>pheA</i>	This study
BT7P	BL21(DE3) <i>trp</i> promoter::T7 promoter	This study
B-4HBT-1	BL21 (DE3) harboring pC-hbad-CaSHT	This study
B-4HBT-2	BL21 (DE3) harboring pC-hbad-CaSHT and pE-PsTyDC-ubiC	This study
B-4HBT-3	BL21 (DE3) harboring pACYCDuet-1, pC-hbad-CaSHT, and pE-PsTyDC-ubiC	This study
B-4HBT-4	BL21 (DE3) harboring pA-aroG ^f -tyrA ^f , pC-hbad-CaSHT, and pE-PsTyDC-ubiC	This study
B-4HBT-5	BL21 (DE3) harboring pA-aroG ^f -ppsA-tktA-tyrA ^f , pC-hbad-CaSHT, and pE-PsTyDC-ubiC	This study
B-4HBT-6	BL21 (DE3) harboring pA-aroL-aroG ^f -ppsA-tktA-tyrA ^f , pC-hbad-CaSHT, and pE-PsTyDC-ubiC	This study
B-4HBT-7	B-tyrR/pheA harboring pA-aroL-aroG ^f -ppsA-tktA-tyrA ^f , pC-hbad-CaSHT, and pE-PsTyDC-ubiC	This study
B-4HBT-8	B-trpEG/pheA harboring pA-aroL-aroG ^f -ppsA-tktA-tyrA ^f , pC-hbad-CaSHT, and pE-PsTyDC-ubiC	This study
B-2-HBT-1	BL21 (DE3) harboring pC-hbad and pE-OsHCT	This study
B-2-HBT-2	BL21 (DE3) harboring pC-hbad and pE-OsHCT-BaTDC	This study
B-2-HBT-3	BL21 (DE3) harboring pACYCDuet-1, pC-hbad-entC-pchB, and pE-OsHCT-BaTDC	This study
B-2-HBT-4	B-tryR harboring pACYCDuet-1, pC-hbad-entC-pchB, and pE-OsHCT-BaTDC	This study
B-2-HBT-5	BT7P harboring pACYCDuet-1, pC-hbad-entC-pchB, and pE-OsHCT-BaTDC	This study
B-2-HBT-6	BT7P harboring pA-aroL-aroG ^f -ppsA-tktA, pC-hbad-entC-pchB, and pE-OsHCT-BaTDC	This study

and Easy Conditional Knockout Kit (Gene Bridges, Germany). LB medium containing 50 µg/ml kanamycin was used to select the positive colonies. Positive clones were verified by colony PCR with two primers, 5'-CTGGCGTCAGGCAGCCATCG-3' and 5'-CCAGCAGGGCTTCGCCGTTG-3'.

Synthesis and Analysis of Reaction Products

To synthesize 4-HBT from 4-hydroxybenzoic acid and tyramine or 2-HBT from 2-hydroxybenzoic acid and tryptamine, *E. coli* strain B-4HBT-1 or B-2-HBT-1 was prepared [15] and 100 µM substrates were used. For the synthesis of 4-HBT and 2-HBT from

glucose, *E. coli* strains were prepared as described before [17]. Briefly, transformants were grown in LB medium containing suitable antibiotics at 37°C for 18 h. The culture was inoculated into fresh medium containing suitable antibiotics at 37°C for 4 h. To synthesize 4-HBT from glucose, the cells were grown until $OD_{600} = 1$ and were harvested and resuspended in M9 containing 1% yeast extract, 2% glucose, 1 mM isopropyl β -D-thiogalactopyranoside (IPTG) and 50 μ g/ml antibiotics. The cells were grown at 30°C with shaking for 24 h and extracted from ethyl acetate. To synthesize 2-HBT from glucose, the cells were grown until $OD_{600} = 0.8$ and IPTG was added to a final concentration of 1 mM and the culture was incubated at 18°C for 16 h. The cells were grown until $OD_{600} = 3$ and were harvested and resuspended in the same medium for the synthesis of 4-HBT. The cells were grown at 30°C with shaking for 24 h and extracted from ethyl acetate.

The reaction products were analyzed using high-performance liquid chromatography (HPLC; [15]) and nuclear magnetic resonance spectroscopy (NMR) [18]. These reaction products were purified from the culture filtrate with ethyl acetate followed by evaporation of the organic layer. The resulting sample was further purified using HPLC and the structure of purified compound was determined using NMR. The proton NMR data of the synthesized compounds were as follows; 4-Hydroxybenzoyl tyramine; ^1H NMR (methanol- d_4 , 400MHz): δ 2.78 (2H, t, $J = 7.5$ Hz, H- α), 3.49 (2H, d, $J = 7.5$ Hz, H- β), 6.70 (4H, m, H-3'/5', H-3'/5'), 7.05 (2H, d, $J = 8.4$ Hz, H-2'/6'), and 7.59 (2H, d, $J = 8.7$ Hz, H-2'/6'). N-2-Hydroxybenzoyl tryptamine; ^1H NMR (acetone- d_6 , 400MHz): δ 3.07 (2H, t, $J = 7.5$ Hz, H- α), 3.72 (2H, t, $J = 7.5$ Hz, H- β), 6.68 (1H, dd, $J = 7.9, 7.6$ Hz, H-5'), 6.87 (1H, d, $J = 8.4$ Hz, H-3'), 7.01 (1H, dd, $J = 7.9, 7.6$ Hz, H-5), 7.09 (1H, dd, $J = 8.2, 7.6$ Hz, H-6), 7.21 (1H, s, H-2), 7.26 (1H, dd, $J = 8.4, 7.6$ Hz, H-4'), 7.37 (1H, d, $J = 8.2$ Hz, H-7), 7.65 (1H, d, $J = 7.9$ Hz, H-4), and 7.83 (1H, d, $J = 7.9$ Hz, H-6').

Results and Discussion

Synthesis of 4-Hydroxybenzoyl Tyramine

4-HBT is a conjugate of 4-HBA and tyramine. In order to form an amide bond, activation of 4-HBA by the

attachment of CoA is a prerequisite. Conjugation of 4-hydroxybenzoyl-CoA and tyramine is further mediated by another enzyme. Hbad from *R. palustris* is known to attach CoA to 4-hydroxybenzoic acid [19] and amide formation between tyramine and 4-hydroxybenzoyl-CoA was tested using benzyl alcohol *O*-acetyltransferase (BEAT). We tested the acetyltransferases from *C. annuum* (CaSHT). Two genes, *hbad* and *CaSHT* (pC-hbad-CaSHT), were transformed in *E. coli*. The resulting transformant (B-4HBT-1) was used to examine the synthesis of 4-hydroxybenzoyl tyramine by feeding 4-HBA and tyramine. HPLC analysis of the culture filtrate revealed a new peak, which had a different retention time from that of 4-HBA. Molecular mass of this compound was 257.11 Da, which is the predicted molecular mass of 4-HBT. Furthermore, the structure of this compound was confirmed using proton NMR (Materials and Methods).

We also tested other hydroxybenzoic acids as substrates (benzoic acid, 2-HBA, 3-hydroxybenzoic acid [3-HBA], 4-HBA, and 3,4-dihydroxybenzoic acid [3,4-DHBA]), with either tryptamine or tyramine. Among 10 combinations, five benzoic acid derivatives with two amines (tryptamine and tyramine), and five conjugates (benzoyl tyramine, 4-hydroxybenzoyl tyramine, benzoyl tryptamine, 4-hydroxybenzoyl tryptamine, and 2-hydroxybenzoyl tryptamine) were synthesized. Since tyramine is an acyl group acceptor, 4-HBA was a better substrate than benzoic acid. On the other hand, with tryptamine as an acceptor, benzoic acid was the best donor followed by 4-HBA, 2-HBA, and 3-HBA.

We designed a pathway to synthesize 4-hydroxybenzoyl tyramine without feeding 4-HBA and tyramine. Tyramine was synthesized by decarboxylation of tyrosine using tyrosine decarboxylase from *P. somniferum* (*PsTyDC*). 4-HBA is synthesized by chorismate pyruvate-lyase (*ubiC*) from chorismate. Since *E. coli* contains *ubiC* gene, it can synthesize 4-HBA. However, in order to enhance the synthesis of

Fig. 2. Synthesis of 4-hydroxybenzoyl tyramine in *E. coli* strain B-4HBT-2.

A, standard 4-hydroxybenzoic acid (S); B, the reaction products from the strain B-4HBT-2. S is likely to be 4-hydroxybenzoic acid and P is 4-hydroxybenzoyl tyramine.

Fig. 3. Synthesis of 4-hydroxybenzoyl tyramine in *E. coli* expressing different shikimate pathway genes (A) and in *E. coli* mutants (B).

4-HBA, *ubiC* was overexpressed. Both *PsTyDC* and *ubiC* were transformed into B-4HBT-1 and the resulting transformant (B-4HBT-2) was tested for the synthesis of 4-hydroxybenzoyl tyramine. As shown in Fig. 2B, synthesis of 4-hydroxybenzoyl tyramine was indeed observed. We also observed unreacted 4-hydroxybenzoic acid, which indicated that the amount of tyramine was less than that of 4-HBA in *E. coli*. Synthesis of chorismate, the substrate of 4-HBA, did not undergo feedback inhibition, whereas synthesis of tyrosine, the substrate of tyramine, did [20]. In an attempt to synthesize more endogenous substrate, tyrosine, two genes, feedback-free versions of wild type, *aroG^f* and *tyrA^f*, were overexpressed. For chorismate synthesis, genes in the shikimate pathway were also overexpressed. Four constructs, having various combinations of genes of the shikimate pathway of *E. coli*, were prepared and tested for the production of 4-HBT. Overexpression of *aroG^f* and *tyrA^f* (strain B-4HBT-4) enhanced the yield of 4-HBT from 58.5 mg/l to 98.3 mg/l (Fig. 3A). Overexpression of two additional genes, *tktA* and *ppsA* (B-4HBT-5), both of which provide erythrose 4-phosphate and phosphoenolpyruvate, increased the synthesis of 4-HBT (113.2 mg/l) by probably providing more substrates for AroG. Furthermore, overexpression of *aroL* along with *aroG^f*, *tyrA^f*, *tktA*, and *ppsA* (strain B-4HBT-6) enhanced the synthesis of 4-hydroxybenzoyl tyramine (126.8 mg/l), which was a more than 2-fold increase compared to that in strain B-4HBT-3. Supplementation of chorismate by overexpressing shikimate pathway genes resulted in the increased synthesis of 4-HBT. This indicated that the downstream enzymes in this pathway (*UbiC* (chorismate pyruvate-lyase), *PsTyDC* (tyrosine decarboxylase), *HbaD* (hydroxybenzoate coenzyme A ligase), and *CaSHT* (Serotonin *N*-(hydroxycinnamoyl) transferase)) were not the bottleneck of the pathway.

Next, we used *E. coli* mutant strains to supply more substrates, tyrosine and chorismate, thereby enhancing the synthesis of 4-hydroxybenzoyl tyramine. Two mutants were used for the purpose; *TyrR*/*PheA* (*PheA* competes with *TyrA* for prephenate to synthesize tyrosine) double mutant is already known to synthesize more tyrosine [20]. Since *TrpEG* converts chorismate into anthranilate for the synthesis of tryptophan, and *PheA* converts prephenate into phenylpyruvate for the synthesis of phenylalanine [21, 22], *TrpEG*/*PheA* double mutant would supply more substrates for 4-HBA and tyrosine. As expected, the synthesis of 4-hydroxybenzoyl tyramine was dramatically enhanced in these mutants (Fig. 3B); strain B-4HBT-6, -7, and -8 synthesized 124.0, 176.9, and 214.9 mg/l of 4-HBT, respectively. The *TrpEG*/*PheA* mutant (strain B-4HBT-8) produced approximately 70% more 4-HBT than the wild-type strain (strain B-4HBT-6). *TrpEG*/*PheA* seemed to have provided more of both substrates than the wild type. Using strain B-4HBT-8, we could synthesize approximately

Fig. 4. Synthesis of 4-hydroxybenzoyl tyramine using *E. coli* strain B-4HBT-8.

Fig. 5. Synthesis of *N*-2-hydroxybenzoyl tryptamine from glucose using *E. coli* strain B-2-HBT-3.

A, standard 2-hydroxybenzoic acid (S); B, reaction products from B-2-HBT-3. S is likely to be 2-hydroxybenzoic acid and P is *N*-2-hydroxybenzoyl tryptamine.

259.3 mg/l of 4-hydroxybenzoyl tyramine after a 24 h reaction. 4-HBA was also monitored and its accumulation was observed when the synthesis of 4-HBT reached almost the maximum (Fig. 4).

Synthesis of *N*-2-Hydroxybenzoyl Tryptamine

2-HBT is a conjugate of 2-HBA and tryptamine. Two enzymatic reactions were necessary for its synthesis; attachment of CoA to 2-HBA and amide formation between 2-hydroxybenzoyl-CoA and tryptamine. Hydroxybenzoic acid-CoA ligase (*hbad*) is known to catalyze the first reaction [19]. To find a gene encoding amide formation, we used BAHD *N*-acyltransferases from rice [23]. One gene (*OsHCT*), along with *hbad*, was transformed into *E. coli*. 2-HBA and tryptamine were added to the culture medium of *E. coli* carrying *OsHCT* and *hbad* (*E. coli* B-2-HBT-1 in Table 1). An HPLC diagram showed that the *E. coli* B-2-HBT-1 synthesized a new product, which had the same molecular mass as 2-HBT. Proton NMR confirmed the synthesized compound as 2-HBT.

As an acyl group donor, benzoic acid, 2-HBA, 3-HBA, 4-HBA, and 3,4-DHBA were used. *OsHCT* was found to use four of the benzoic acids, except 3,4-DHBA, to synthesize the corresponding tryptamines. *OsHCT* could use tyramine as an acyl group acceptor with 3-HBA and benzoic acid as donors. However, it could not use 4-HBA as a donor (data not shown).

Next, we attempted to synthesize 2-HBT by feeding only 2-HBA. Since *E. coli* has endogenous tryptophan, we employed the tryptophan decarboxylase from *Bacillus atrophaeus* to synthesize tryptamine from tryptophan [16]. The resulting transformant (B-2-HBT-2) also synthesized 2-HBT when 2-HBA was supplied (data not shown).

Finally, to synthesize 2-HBT from glucose, the 2-HBA synthesis pathway was introduced into *E. coli*. Two genes,

entC from *E. coli* and *pchB* from *Pseudomonas fluorescens*, converted chorismate to 2-HBA. *E. coli* strain B-2-HBT-2 was transformed with these two genes and the resulting transformant (*E. coli* B-2-HBT-3) was tested for the synthesis of 2-HBT. As expected, 2-HBT was indeed synthesized by *E. coli* B-2-HBT-3 (Fig. 5). 2-HBA was also observed, indicating the necessity of supply of tryptophan for synthesizing more 2-HBT. We tested two mutant strains; the first mutant had a deletion in the negative transcription regulator encoded by the *tyrR* gene, which was activated by tryptophan [24, 25]. The *trp* promoter of *trp* operon in the second mutant (*E. coli* strain BT7P in Table 1) was replaced by a stronger promoter T7. We prepared two strains (*E. coli* B-2-HBT-4 and *E. coli* B-2-HBT-5) and examined the synthesis of 2-HBT. *E. coli* B-2-HBT-5 synthesized the highest amount of 2-HBT (192.4 mg/l), followed by *E. coli* B-2-HBT-4 (183.9 mg/l) and *E. coli* B-2-HBT-3 (157.1 mg/l) (Fig. 6). The 2-HBA of these three strains was 55.2, 78.6, and 36.8 mg/l, respectively. *E. coli*

Fig. 6. Synthesis of *N*-2-hydroxybenzoyl tryptamine in different *E. coli* strains.

Fig. 7. Synthesis of N-2-hydroxybenzoyl tryptamine using *E. coli* strain B-2-HBT-6.

BT7P supplied more tryptophan for the synthesis of 2-HBT. TyrR is a regulator of shikimate pathway and therefore, its deletion led to enhanced synthesis of chorismate, which could convert into both 2-HBA and tryptophan. Therefore, *E. coli* B-tyrR strain increased the synthesis of not only 2-HBT but also 2-HBA. We also introduced the construct pA-aroL-aroG^f-ppsA-*tktA*, which was effective for the production of 4-HBT, into strain B-2-HBT-5. The resulting strain (B-2-HBT-6) produced a little more 2-HBT (227.8 mg/l) and even more 2-HBA (138.0 mg/l) than strain B-2-HBT-5. This result showed that a balanced supply of both substrates is critical for the final yield; by overexpressing four genes of the shikimate pathway, sufficient amounts of 2-HBA were provided. But, the final yield of 2-HBT was not increased due to the tryptophan. Therefore, the bottleneck of 2-HBT synthesis was the synthesis of tryptophan.

Using B-2-HBT-6, the synthesis of 2-HBT was monitored for 24 h (Fig. 7). 2-HBT yield continued to increase until 15 h (227.2 mg/l) and then remained almost unchanged. However, 2-HBA started to accumulate rapidly from 15 h, indicating that tryptophan was the limiting factor.

We synthesized two hydroxybenzoic acid-amine conjugates. To maximize the synthesis of both products, we overexpressed four genes (*aroG^f*, *tktA*, *ppsA*, and *aroL*) to ensure the supply of increased substrates. In addition, mutant strains were used during the synthesis of 4-HBT to increase two substrates, chorismate and tyrosine, more effectively. More tryptophan was supplied by replacing the *trp* operon promoter. Fine tuning of substrates is, therefore, a critical issue in increasing the final yield.

Two HCTs, CaSHT, and OsHCT, used diverse substrates. CaSHT used diverse hydroxycinnamic acid derivatives (*o*-coumaric acid, *m*-coumaric acid, *p*-coumaric acid, caffeic acid, ferulic acid, etc.) and various amines including

tryptamine, serotonin, and tyramine [15, 16]. It was shown that CaSHT expanded its substrates to the benzoic acid derivatives. OsHCT not only used tryptamine but also tyramine. Promiscuity of CaSHT and OsHCT was useful in synthesizing a variety of diverse amine conjugates, thereby enabling novel biological activities of the synthesized compounds to be explored.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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