Bacterial synthesis of \(N\)-hydroxycinnamoyl phenethylamines and tyramines

Geun Young Sim\(^1\), So-Mi Yang\(^1\), Bong Gyu Kim\(^2\) and Joong-Hoon Ahn\(^1\)*

Abstract

Background: Hydroxycinnamic acids (HCAs) including cinnamic acid, \(p\)-coumaric acid, caffeic acid, and ferulic acid, are \(C_6–C_3\) phenolic compounds that are synthesized via the phenylpropanoid pathway. HCAs serve as precursors for the synthesis of lignins, flavonoids, anthocyanins, stilbenes and other phenolic compounds. HCAs can also be conjugated with diverse compounds including quinic acid, hydroxyl acids, and amines. Hydroxycinnamoyl (HC) amine conjugates such as \(N\)-HC tyramines and \(N\)-HC phenethylamines have been considered as potential starting materials to develop antiviral and anticancer drugs.

Results: We synthesized \(N\)-HC tyramines and \(N\)-HC phenethylamines using three different approaches in *Escherichia coli*. Five \(N\)-HC phenethylamines and eight \(N\)-HC tyramines were synthesized by feeding HCAs and phenethylamine or tyramine to *E. coli* harboring 4CL (encoding 4-coumarate CoA ligase) and either SHT (encoding phenethylamine \(N\)-HC transferase) or THT (encoding tyramine \(N\)-HC transferase). Also, \(N\)-(\(p\)-coumaroyl) phenethylamine and \(N\)-(\(p\)-coumaroyl) tyramine were synthesized from \(p\)-coumaric acid using *E. coli* harboring an additional gene, PDC (encoding phenylalanine decarboxylase) or TDC (encoding tyrosine decarboxylase). Finally, we synthesized \(N\)-(\(p\)-coumaroyl) phenethylamine and \(N\)-(\(p\)-coumaroyl) tyramine from glucose by reconstructing the metabolic pathways for their synthesis in *E. coli*. Productivity was maximized by optimizing the cell concentration and incubation temperature.

Conclusions: We reconstructed the metabolic pathways for synthesis of \(N\)-HC tyramines and \(N\)-HC phenethylamines by expressing several genes including 4CL, TST or SHT, PDC or TDC, and TAL (encoding tyrosine ammonia lyase) and engineering the shikimate metabolic pathway to increase endogenous tyrosine concentration in *E. coli*. Approximately 101.9 mg/L \(N\)-(\(p\)-coumaroyl) phenethylamine and 495.4 mg/L \(N\)-(\(p\)-coumaroyl) tyramine were synthesized from \(p\)-coumaric acid. Furthermore, 152.5 mg/L \(N\)-(\(p\)-coumaroyl) phenethylamine and 94.7 mg/L \(N\)-(\(p\)-coumaroyl) tyramine were synthesized from glucose.

Keywords: Hydroxycinnamate, Hydroxycinnamate amine, Metabolic engineering

Background

Plants synthesize phenolic compounds through the phenylpropanoid pathway. Hydroxycinnamic acids (HCAs) serve as precursors for the synthesis of lignins, flavonoids, anthocyanins, stilbenes and other phenolic compounds [1]. Hydroxycinnamates can be conjugated with diverse compounds. In the formation of hydroxycinnamate conjugates, hydroxycinnamates such as cinnamic acid, \(p\)-coumaric acid, caffeic acid, and ferulic acid can serve as donors, while diverse chemicals including quinic acid, hydroxyl acids (maleic acid and tartaric acid), amino compounds (aromatic amino acids, choline, and anthranilic acids), and polysaccharides (glycerol, anthocyanin glycosides, flavonoid glycosides, and terpene glycosides) can be used as acceptors [2].

Hydroxycinnamoyl (HC) amides are an example of hydroxycinnamate conjugates, in which tyramine, phenethylamine, serotonin, antranilate, tryptamine, or...
tyramines, the two amino acids phenylalanine and tyrosine are critical factors for the synthesis of phenylalanine ammonia lyase (TAL) via the phenylpropanoid pathway [22]. Therefore, the endogenous concentrations of tyrosine and phenylalanine are also observed during the synthesis of N-(p-coumaroyl) phenethylamine. N-(p-coumaroyl) phenethylamine was isolated from Arabidopsis thaliana [9].

Many plant metabolites have been used as starting molecules to develop new medicines [10, 11]. HC amides were originally known as antioxidants, like other plant phenolic compounds. However, recent studies have shown that HC amides could be developed into new candidate medicines. For example, hydroxycinnamate amide served as a backbone for the synthesis of antiviral compounds [12]. Analogs of N-HC phenalkylamines are inhibitors of tyrosinase in human melanocytes, which has the potential to treat pigmentation-related disorders [13]. N-HC tyramine showed anti-proliferative effects on cancer cells [14] and selectively induced the apoptosis of cancer cells [15].

For the synthesis of N-HC phenethylamines and N-HC tyramines, the two amino acids phenylalanine and tyrosine, respectively, serve as the key substrates. Phenethylamine and tyramine are synthesized from phenylalanine and tyrosine by phenylalanine decarboxylase (PDC) and tyrosine decarboxylase (TDC), respectively. PDC has been isolated and characterized from tomato [16] and TDC has been isolated from several plants including parsley, Papaver somniferum, Arabidopsis thaliana, and Catharanthus roseus [17–21]. PDC and TDC show catalytic activity for both phenylalanine and tyrosine as substrates, but have been named depending on their substrate preference. On the other hand, hydroxycinnamic acids are also synthesized from these two amino acids by phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL) via the phenylpropanoid pathway [22]. Therefore, the endogenous concentrations of tyrosine and phenylalanine are critical factors for the synthesis of N-HC tyramines and N-HC phenethylamines in Escherichia coli.

The formation of conjugates between HCs and phenethylamine or tyramine can be mediated by HC transferases (HCTs). HCTs use HC-CoA as the HC donor and various compounds including phenethylamine and tyramine as HC acceptors [23]. HC-CoA: tyramine (HC) transferase (THT) catalyzes the formation of N-HC tyramines; the corresponding gene has been cloned from Capsicum annuum [24]. HC-CoA: serotonin N-(HC) transferase (SHT) from C. annuum was shown to catalyze the formation of N-HC phenethylamines although it demonstrated higher affinity for serotonin than for phenethylamine [25]. Using these genes, it was possible to reconstruct the pathways for the synthesis of N-(p-coumaroyl) phenethylamine and N-(p-coumaroyl) tyramine in E. coli (Fig. 1).

In this report, we synthesized N-HC tyramines and N-HC phenethylamines from tyrosine and phenylalanine using metabolically engineered E. coli. The shikimate pathway of E. coli which leads to the production of phenylalanine, tyrosine, and tryptophan, is well-characterized and strategies to increase the intracellular concentrations of tyrosine and phenylalanine are also known. [26]. Using engineered E. coli strains, we were able to synthesize N-HC phenethylamines and N-HC tyramines from glucose as well as from HCs.

Results
Production of N-HC phenethylamines and N-HC tyramines in E. coli
N-HC phenethylamines and N-HC tyramines were synthesized from HC and phenethylamine or tyramine. Two enzymatic reactions were required for the synthesis of these compounds. The first reaction was activation of HCA by attaching CoA to HCA to form HC-CoA, which is catalyzed by 4-coumarate CoA:ligase (4CL), and the second reaction was formation of the conjugate between HC-CoA and tyramine or phenethylamine, which is mediated by HCT. We cloned two genes, 4CL from Oryza sativa and SHT from C. annuum, into an E. coli expression vector for the synthesis of N-HC phenethylamine. The resulting E. coli strain was named HP-1. For the synthesis of N-HC tyramines, we used strain HT-1, which expresses 4CL from O. sativa and THT from C. annuum. The formation of the reaction products after feeding strain HP-1 or strain HT-1 with p-coumaric acid and phenethylamine or tyramine is shown in Fig. 2. Peaks of UV absorbance (P1 in Fig. 2a and P2 in Fig. 2b) obtained by high-pressure liquid chromatography (HPLC) were confirmed as N-(p-coumaroyl) phenethylamine and N-(p-coumaroyl) tyramine by mass spectrometry (data not shown). In addition, the structures of N-(p-coumaroyl) phenethylamine and N-(p-coumaroyl) tyramine were determined using nuclear magnetic resonance spectroscopy (NMR) (see “Methods”). We also observed the formation of a small amount of N-(p-coumaroyl) tyramine (peak at 7.8 min in Fig. 2a) during the formation of N-(p-coumaroyl) phenethylamine. Formation of N-(p-coumaroyl) phenethylamine was also observed during the synthesis of N-(p-coumaroyl) tyramine (peak at 10.1 min in Fig. 2b). An explanation for this is that yeast extract used in the growth medium

Sim et al. Microb Cell Fact (2015) 14:162
Twelve HCA were tested to determine the substrate range of 4CL, SHT and THT using E. coli strains HP-1 and HT-1 (Table 1). Each HCA was fed to HP-1 along with phenethylamine. The strain HP-1 produced reaction products with five of HCs. Among them, 3-methoxy cinnamic acid was the best substrate, followed by caffeic acid, cinnamic acid, p-coumaric acid, and ferulic acid. For the formation of N-HC tyramines, strain HT-1 was used. Eight of twelve HCAAs formed conjugates with tyramine. Again, 3-methoxy cinnamic acid was the best substrate, followed by ferulic acid, m-coumaric acid, cinnamic acid, caffeic acid, o-coumaric acid, p-coumaric acid, and 4-methoxy-cinnamic acid (Table 2). Among five naturally occurring HCAAs (cinnamic acid, p-coumaric acid, caffeic acid, ferulic acid, and sinapic acid), all of them except sinapic acid led to the formation of either N-HC phenethylamines or N-HC tyramines. These results indicate that the size of each HC was a determining factor in whether they could act as substrates for the formation of the corresponding N-HC phenethylamines or N-HC tyramines. When cinnamic acids containing more than two methoxy groups were used as substrates, neither N-HC phenethylamines nor N-HC tyramines were formed.

Synthesis of N-(p-coumaroyl) phenethylamine in E. coli
Among the several reaction products, we decided to synthesize N-(p-coumaroyl) phenethylamine and N-(p-coumaroyl) tyramine by feeding E. coli with only p-coumaric acid. For this, either a decarboxylase that can convert phenylalanine into phenethylamine or one that can convert tyrosine into tyramine was introduced into E. coli to synthesize N-(p-coumaroyl) phenethylamine and N-(p-coumaroyl) tyramine.
the E. coli strains HP-1 and HT-1. We engineered strain HP-2 to synthesize N-(p-coumaroyl) phenethylamine by transforming E. coli with PDC along with SHT and 4CL and then feeding them p-coumaric acid. Analysis of the cultures from strain HP-2 using HPLC showed a peak with the same retention time as that of N-(p-coumaroyl) phenethylamine and its structure as that compound was confirmed by MS and NMR (data not shown). Therefore, we decided to use the HP-2 strain for optimization of the production of N-(p-coumaroyl) phenethylamine.

First, we tested the effect of initial cell density. The cell density of HP-2 was adjusted to an OD₆₀₀ of 1, 3, 5, or 7. The production of N-(p-coumaroyl) phenethylamine increased from 22.8 mg/L at OD₆₀₀ = 1, 55.7 mg/L at OD₆₀₀ = 3, and 59.6 mg/L at OD₆₀₀ = 5, but decreased to 51.6 mg/L at OD₆₀₀ = 7. The optimal incubation temperature was determined at the optimized cell density of OD₆₀₀ = 5. HP-2 grown at 25, 30, or 37 °C produced 43.1, 59.6, or 48.2 mg/L N-(p-coumaroyl) phenethylamine, respectively. The initial p-coumaric acid concentration was then optimized with the optimized cell density (OD₆₀₀ = 5) and incubation temperature (30 °C). The strain HP-2 was fed with 0.2, 0.4, 0.6, 0.8, or 1.0 mM of p-coumaric acid, and the production of N-(p-coumaroyl) phenethylamine was examined. HP-2 converted 81.8 % of 0.2 mM p-coumaric acid into N-(p-coumaroyl) phenethylamine, producing 43.7 mg/L N-(p-coumaroyl) phenethylamine. When the cells were fed with 0.4 mM p-coumaric acid, the conversion rate was 80.4 %, producing 85.9 mg/L N-(p-coumaroyl) phenethylamine. At higher p-coumaric acid concentrations, the conversion rate of p-coumaric acid was less than 50 %

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

| Plasmids or E. coli strain | Relevant properties or genetic marker(s) | Source or references |
|---------------------------|------------------------------------------|----------------------|
| pACYCDuet | P15A ori, Cm<sup>+</sup> | Novagen |
| pCDFDuet | CloDE13 ori, Str<sup>+</sup> | Novagen |
| pEDuet | f1 ori, Amp<sup>+</sup> | Novagen |
| pA-SeTAL | pACYCDuet carrying TAL from *Saccharothrix espanaensis* | [31] |
| pA-aroG<sup>+</sup>-SeTAL-tyrA | pACYCDuet carrying TAL from *S. espanaensis*, aroG and tyrA from *Escherichia coli* | [31] |
| pA-aroG<sup>+</sup>fbr- SeTAL-tyrAfbr | pACYCDuet carrying TAL from *S. espanaensis*, aroG<sup>+</sup>fbr, and tyrA<sup>+</sup>fbr from *E. coli* | |
| pE-PDC | pETDuet carrying PDC from *Pseudomonas putida* | |
| pC-Os4CL-SHT | pCDFDuet carrying 4CL from *Oryza sativa* and SHT from *Capsicum annuum* | |
| pC-Os4CL-THT | pCDFDuet carrying 4CL from *O. sativa* and THT from *C. annuum* | This study |
| pC-TDC-Os4CL-THT | pCDFDuet carrying TDC from *Papaver somniferum*, 4CL from *O. sativa*, and THT from *C. annuum* | This study |
| Strains | | |
| BL21(DE3) | F<sup>−</sup> ompT hisD<sub>5</sub>(g<sub>2</sub>m<sub>5</sub>) gal dcm ion (DE3) | Novagen |
| BtyR | BL21(DE3) ΔtyrR | [31] |
| BtyR-trpD | BL21(DE3) ΔtyrR/ΔtrpD | This study |
| HP-1 | BL21 harboring pC-Os4CL-SHT | This study |
| HP-2 | BL21 harboring pC-Os4CL-SHT and pE-PDC | This study |
| HP-3 | BL21 harboring pC-Os4CL-SHT, pA-SeTAL, and pE-PDC | This study |
| HP-4 | BL21 harboring pC-Os4CL-SHT, pA-aroG<sup>+</sup>-SeTAL-tyrA, and pE-PDC | This study |
| HP-5 | BL21 harboring pC-Os4CL-SHT, pA-aroG<sup>+</sup>-SeTAL-tyrA<sup>+</sup>, and pE-PDC | This study |
| HP-6 | BtyR harboring pC-Os4CL-SHT, pA-aroG<sup>+</sup>-SeTAL-tyrA<sup>+</sup>, and pE-PDC | This study |
| HP-7 | BtyR-trpD harboring pC-Os4CL-SHT, pA-aroG<sup>+</sup>-SeTAL-tyrA<sup>+</sup>, and pE-PDC | This study |
| HT-1 | BL21 harboring pC-Os4CL-THT | This study |
| HT-2 | BL21 harboring pC-TDC-Os4CL-THT | This study |
| HT-3 | BL21 harboring pC-TDC-Os4CL-THT and pA-aroG-tyrA | This study |
| HT-4 | BL21 harboring pC-TDC-Os4CL-THT and pA-aroG<sup>+</sup>-tyrA<sup>+</sup> | This study |
| HT-5 | BL21 harboring pC-TDC-Os4CL-THT and pA-SeTAL | This study |
| HT-6 | BL21 harboring pC-TDC-Os4CL-THT and pA-aroG<sup>+</sup>-SeTAL-tyrA | This study |
| HT-7 | BL21 harboring pC-TDC-Os4CL-THT and pA-aroG<sup>+</sup>-SeTAL-tyrA<sup>+</sup> | This study |
| HT-8 | BtyR-ph<sub>E</sub>A harboring pC-TDC-Os4CL-THT and pA-aroG<sup>+</sup>-SeTAL-tyrA<sup>+</sup> | This study |
| HT-9 | BtyR-trpD harboring pC-TDC-Os4CL-THT and pA-aroG<sup>+</sup>-SeTAL-tyrA<sup>+</sup> | This study |
and the final yields of N-(p-coumaroyl) phenethylamine were 78.5, 74.0 and 59.6 mg/L, respectively, which were much lower than the yield at 0.4 mM p-coumaric acid. Using the optimized initial concentration of p-coumaric acid (0.4 mM), we monitored the production of N-(p-coumaroyl) phenethylamine and found that approximately 101.9 mg/L N-(p-coumaroyl) phenethylamine was produced after 12 h, after which the yield decreased until 24 h (data not shown). Approximately 95.3 % of p-coumaric acid was converted into N-(p-coumaroyl) phenethylamine.

N-(p-Coumaroyl) phenethylamine is a conjugate of p-coumaric acid and phenethylamine. p-Coumaric acid is synthesized from tyrosine by TAL and phenethylamine is synthesized from phenylalanine by PDC. Therefore, the intracellular concentrations of both tyrosine and phenylalanine are critical for the final yield of N-(p-coumaroyl) phenethylamine. Tyrosine and phenylalanine are synthesized through the shikimate pathway. Two genes, aroG and tyrA, were overexpressed in order to increase the amount of tyrosine and phenylalanine. The gene aroG, which encodes 2-dehydro-3-deoxyphosphohexonate aldolase, catalyzes the first reaction in the shikimate pathway. The gene tyrA, which encodes chorismate mutase/prephenate dehydrogenase, uses chorismate to make prephenate, which is used for the synthesis of phenylalanine and tyrosine. Both the AroG and TyrA proteins are inhibited by their final products (phenylalanine and tyrosine, respectively). Therefore, the feed back inhibition-free versions of aroG (aroG<sub>fbr</sub>) and tyrA (tyrA<sub>fbr</sub>) were used [27]. The production of N-(p-coumaroyl) phenethylamine was examined in three E. coli strains (HP-3, HP-4, and HP-5). HP-5 produced the highest amount of N-(p-coumaroyl) phenethylamine at approximately 46.1 mg/L, followed by HP-4 (29.6 mg/L), and HP-3 (14.6 mg/L) (Fig. 3a). Therefore, the tyrosine concentration in E. coli was directly related to the yield of N-(p-coumaroyl) phenethylamine. Next, we tested different E. coli mutants, which are expected to increase the production of tyrosine. The tyrR deletion mutant was the first mutant strain. TyrR is a transcription factor that is inhibited by tyrosine and phenylalanine. Deletion of trpD, which encodes the enzyme for the synthesis of tryptophan from chorismate, can result in higher substrate availability for the production of both tyrosine and phenylalanine. Therefore, two mutant strains,

| Substrate           | Structure | N-HC phenethylamine (relative activity %)<sup>2</sup> | N-HC tyramine (relative activity %) |
|---------------------|-----------|------------------------------------------------------|-------------------------------------|
| p-Coumaric acid     | ![p-Coumaric acid](image) | 36                                                   | 52                                  |
| m-Coumaric acid     | ![m-Coumaric acid](image) | 0                                                   | 86                                  |
| α-Coumaric acid     | ![α-Coumaric acid](image) | 0                                                   | 66                                  |
| Caffeic acid        | ![Caffeic acid](image) | 43                                                   | 66                                  |
| Ferulic acid        | ![Ferulic acid](image) | 35                                                   | 92                                  |
| Cinnamic acid       | ![Cinnamic acid](image) | 40                                                   | 85                                  |
| 3-Methoxycinnamic acid | ![3-Methoxycinnamic acid](image) | 100                                               | 100                                 |
| 4-Methoxycinnamic acid | ![4-Methoxycinnamic acid](image) | 0                                                   | 41                                  |

<sup>1</sup> 3,4-dimethoxycinnamic acid, 2,4-dimethoxycinnamic acid, sinapic acid, and 3,4,5-trimethoxycinnamic acid were tested but did not serve as substrates for either enzyme

<sup>2</sup> Relative activity was calculated by denoting the activity of the best substrate as 100 %
B-tryR (HP-6) and B-tyrR-typD (HP-7), both of which were transformed with the genes for \(N\)-(\(p\)-coumaroyl) phenethylamine, were used to examine the production of \(N\)-(\(p\)-coumaroyl) phenethylamine. Strain HP-7 produced 104.6 mg/L \(N\)-(\(p\)-coumaroyl) phenethylamine and HP-6 produced 92.9 mg/L (Fig. 3b). This result indicated that the final yield of \(N\)-(\(p\)-coumaroyl phenethylamine) was increased when more precursors for both tyrosine and phenylalanine are available.

Using the strain HP-7, the incubation temperature and the initial cell density were optimized. HP-7 was grown at 18, 25, or 30 °C. The highest production of \(N\)-(\(p\)-coumaroyl) phenethylamine occurred in cells grown at 25 °C (106.1 mg/L), while the yields at 18 and 30 °C were approximately 90.8 and 88.5 mg/L, respectively. The optimal initial cell density was also examined. The cell density of HP-7 was adjusted with the culture medium to OD\(_{600}\) = 0.5, 1, or 1.5, and the production of \(N\)-(\(p\)-coumaroyl) phenethylamine was examined at the optimized temperature of 25 °C. The yield of \(N\)-(\(p\)-coumaroyl) phenethylamine was decreased from 136.1 mg/L at OD\(_{600}\) = 0.5, 106.1 mg/L at OD\(_{600}\) = 1.0 and 91.0 mg/L at OD\(_{600}\) = 1.5. Using the optimized incubation temperature and cell density of strain HP-7, the production of \(N\)-(\(p\)-coumaroyl) phenethylamine was monitored for 36 h. The production of \(N\)-(\(p\)-coumaroyl) phenethylamine was observed at 6 h and continued to increase until 15 h, when production was maximized. The yield of \(N\)-(\(p\)-coumaroyl) phenethylamine at 15 h was 152.5 mg/L. After 15 h, production was decreased (Fig. 4).

**Synthesis of \(N\)-(\(p\)-coumaroyl) tyramine in \textit{E. coli}**

In order to synthesize \(N\)-(\(p\)-coumaroyl) tyramine by feeding with \(p\)-coumaric acid, an additional gene, \textit{TDC} was introduced into HT-1. The resulting strain HT-2 was fed with \(p\)-coumaric acid and analysis of the reaction products using HPLC showed that \(N\)-(\(p\)-coumaroyl) tyramine was synthesized (data not shown). We also tested the optimal initial concentration of \(p\)-coumaric acid and found that the production of \(N\)-(\(p\)-coumaroyl) tyramine was increased up to 2.5 mM \(p\)-coumaric acid. Next, we tested the effect of tyrosine on the production of \(N\)-(\(p\)-coumaroyl) tyramine. Although \(p\)-coumaric acid was externally supplied to \textit{E. coli}, tyramine needed to be synthesized from tyrosine. The strain HT-4, containing the feedback-inhibition free versions of \textit{aroG} and \textit{tyrA} (\textit{aroG} \(^{\beta\text{fr}}\) and \textit{tyrA} \(^{\beta\text{fr}}\)) produced approximately 80.0 mg/L \(N\)-(\(p\)-coumaroyl) tyramine, which is more than was produced by strains HT-2 (48.2 mg/L) and HT-3 (58.9 mg/L). Using strain HT-4, we optimized the incubation temperature and initial cell concentration. The optimal incubation temperature and initial cell concentration were determined to be 30 °C and OD\(_{600}\) = 7, respectively. Using HT-4, approximately 495.4 mg/L \(N\)-(\(p\)-coumaroyl) tyramine was synthesized after 24 h of incubation, after which the production decreased (Fig. 5).

Next, we synthesized \(N\)-(\(p\)-coumaroyl) tyramine from tyrosine. For this, the intracellular concentration of tyrosine is critical. Therefore, we used the same strategies as used for the synthesis of \(N\)-(\(p\)-coumaroyl)
phenethylamine in order to supply more tyrosine. We examined the effects of different constructs and E. coli mutants on the production of N-(p-coumaroyl) tyramine. As expected, the strain HT-7 (36.0 mg/L) produced more tyrosine than the other strains did (HT-6, 6.3 mg/L and HP-5, 2.5 mg/L) (Fig. 6a). Therefore, the tyrosine concentration in E. coli was directly related to the yield of N-(p-coumaroyl) tyramine. Also, the E. coli mutant, BtryR-trpD (HT-9, 57.1 mg/L) produced more N-(p-coumaroyl) tyramine than BtryR-pheA (HT-8, 37.8 mg/L). Again, the intracellular tyrosine concentration was directly related to the final yield of N-(p-coumaroyl) tyramine (Fig. 6b).

In order to optimize the production of N-(p-coumaroyl) tyramine using the strain HT-9, the incubation temperature and initial cell density were optimized. The best temperature was determined to be 30 °C (57.1 mg/L) followed by 25 °C (52.5 mg/L), 37 °C (39.2 mg/L), and 18 °C (22.0 mg/L).

Using the strain HT-9, the incubation temperature and the initial cell density were optimized. HT-9 was grown at 18, 25, 30, or 37 °C. The highest production of p-coumaroyl tyramine occurred in cells grown at 30 °C (57.06 mg/L). The yields at 18, 25 and 37 °C were approximately 21.98, 52.47 and 39.16 mg/L, respectively. The optimal initial cell density was also examined. The production of N-(p-coumaroyl) tyramine was optimized from OD_{600} = 0.5 to OD_{600} = 2 but decreased at OD_{600} = 3.

The production of N-(p-coumaroyl) tyramine was monitored for 36 h with strain HT-9. The production of N-(p-coumaroyl) tyramine was observed at 3 h and continued to increase until 27 h, at which time the production of N-(p-coumaroyl) tyramine was maximized. The yield of N-(p-coumaroyl) tyramine at 27 h was 94.7 mg/L. After 27 h, the production decreased (Fig. 7).

Discussion

Five N-HC phenethylamines and eight N-HC tyramines from 12 HCAs and corresponding amines (phenethylamine and tyramine) were synthesized. Two enzymatic reactions are required to synthesize N-HC phenethylamines or N-HC tyramines in E. coli. The first step is the activation of HC by 4CL which results in the formation of HC-CoA. The second step is the conjugation of HC-CoA to phenethylamine or tyramine, which is catalyzed by either SHT or THT. Based on the results of HT-1 biotransformation, Os4CL used in this study could use at least eight HCAs as substrates (p-coumaric acid, m-coumaric acid, o-coumaric acid, caffeic acid, ferulic acid, cinnamic acid, 3-methoxycinnamic acid, and 4-methoxycinnamic acid). However, HCAs having two methoxy groups did not yield any reaction products. It is not clear whether this result is due to the failure of Os4CL to convert HCA into HC-CoA or due to the formation of conjugates between HC-CoA and tyramine or phenethylamine by THT or SHT. These results also suggested that THT, which was used for the formation of N-HC tyramines, had broader acyl donor specificity than did SHT, which was used for the synthesis of N-HC phenethylamines.

It is not clear whether the different rates of N-HC phenethylamines and N-HC tyramines formation observed can be attributed to the first step or the second step. It is, however, clear that Os4CL used p-coumaric acid or caffeic acid more efficiently than it used ferulic acid [28]. In the formation of N-HC tyamine, N-feruloyl tyramine was synthesized more effectively than N-(p-coumaroyl) tyramine or N-caffeoyl tyramine. These observations suggest that the second reaction catalyzed by THT determines the rate of product formation.

We synthesized N-(p-coumaroyl) phenethylamines and N-(p-coumaroyl) tyramines using three different approaches. Firstly, HC and either phenethylamine or tyramine were used as substrates. Two genes, 4CL and either SHT for N-(p-coumaroyl) phenethylamine biosynthesis or THT for N-(p-coumaroyl) tyramine biosynthesis, were transformed into E. coli and various HC-aminos were successfully synthesized. Secondly, HC
and either phenylalanine or tyrosine were used. For this, an additional gene, either PDC or TDC was introduced to convert phenylalanine or tyrosine into phenethylamine or tyramine, respectively. Thirdly, we synthesized N-(p-coumaroyl) phenethylamine and N-(p-coumaroyl) tyramine from amino acids (phenylalanine and tyrosine, respectively) by introducing TAL into E. coli. In these approaches, the yield of the major product was much higher than that of byproduct(s). It is likely that it is related to the specificity of the enzymes employed in this study. During screening of HCTs using biotransformation, we observed that both SHT and THT were used both phenethylamine and tyramine (Fig. 2), although SHT showed a preference for phenethylamine and THT showed a preference for tyramine. Previous in vitro kinetic data have also shown that SHT has a higher preference for phenethylamine than for tyramine [25]. This is one reason that only a small amount of byproduct is observed. Moreover, the amino acid decarboxylases (PDC and TDC) used in this study showed high substrate specificities. PDC from Pseudomonas putida showed higher substrate specificity for phenylalanine than for tyrosine [29] and TDC from Papaver somniferum showed activity for tyrosine but not for phenylalanine [20]. Furthermore, the TAL we used was previously found to show higher substrate specificity for tyrosine [30] than for phenylalanine. Therefore, byproducts were rarely synthesized. Taken together, using specific enzymes for each reaction step contributed to the synthesis of a specific product with only the formation of a small amount of byproduct(s).

Conclusions
HC-amine conjugates such as N-HC tyramines and N-HC phenethylamines have been considered as potential starting materials for development of antiviral and anticancer drugs. We synthesized N-HC tyramines and N-HC phenethylamines using E. coli harboring 4CL and either THT or SHT. Approximately 101.9 mg/L N-(p-coumaroyl) phenethylamine and 494.5 mg/L N-(p-coumaroyl) tyramine were synthesized from p-coumaric acid using E. coli harboring an additional gene either PDC or TDC. Finally, we synthesized 152.5 mg/L N-(p-coumaroyl) phenethylamine and 94.7 mg/L N-(p-coumaroyl) tyramine from glucose by engineering the shikimate pathway of E. coli and reconstructing the pathway toward synthesis of N-(p-coumaroyl) phenethylamine and N-(p-coumaroyl) tyramine. Productivity was further improved by optimizing the cell concentration and incubation temperature.

Methods
Constructs and E. coli strains
Os4CL was previously cloned in our lab [28] and was subcloned into the EcoRI/NotI sites of pCDFDuet1 (pC-Os4CL). HC-CoA: serotonin N-(HC) transferase (CaSHT) from Capsicum annuum (GenBank accession number AF329463.1) for the synthesis of N-(p-coumaroyl) phenethylamine was cloned using reverse transcription-polymerase chain reaction (RT-PCR). cDNA was isolated from the leaf of C. annuum. PCR was carried out using primers, 5′-ATGATATCGAGCTTCT-TGCTCCTCAACCACCAA-3′ (EcoRV site is underlined.) as a forward primer and 5′-ATCTCGAGCTAACA-GCTTCTGCACCATTATTTCT-3′ (Xhol site is underlined.) as a reverse primer. The resulting PCR product was subcloned into the EcoRV/Xhol sites of pC-Os4CL and named pC-Os4CL-SHT.

PDC from P. putida (GenBank accession number BK006920) was amplified using PCR using genomic DNA as a template using 5′-ATCATATGGTGACCCC-CGAACAAATCCGCC-3′ (NdeI site is underlined.) as a forward primer and 5′-AACCTGAGTCAGCCCTTGAT-CAGT CCTGC-3′ (Xhol site is underlined.) as a reverse primer. The resulting PCR product was subcloned into the NdeI/Xhol sites of pET-Duet1 (Novagen, Madison, WI, USA) and the construct was named pE-PDC. TDC from Papaver somniferum was synthesized after codon optimization for E. coli based on the published sequence (GenBank U08598.1) and subcloned into BamHI/HindIII sites of pCDF. The construct formed was named pC-TDC. Os4CL was amplified with a forward primer containing T7 promoter sequence and HindIII sites and a reverse primer containing a NolI site. The resulting PCR product was subcloned into the HindIII/NolI sites of pC-TDC and the construct was named pC-TDC-Os4CL. Tyramine N-HC transferase (THT) from C. annuum (GenBank accession number AF819700.1) was cloned using RT-PCR. The resulting PCR product was subcloned into the NdeI/Xhol sites of pCDF-TDC-Os4CL and the construct was named pC-TDC-Os4CL-THT.

Synthesis of N-HC phenethylamines and N-HC tyramines
HCAs including p-coumaric acid, m-coumaric acid, o-coumaric acid, caffeic acid, ferulic acid, cinnamic acid, 3-methoxycinnamic acid, 4-methoxycinnamic acid, 3,4-dimethoxycinnamic acid, 2,4-dimethoxycinnamic acid, sinapic acid, and 3,4,5-trimethoxycinnamic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Preference of HCA for the synthesis of N-HC phenethylamines or N-HC tyramines was tested as follows. E. coli strains HP-1 and (Table 1) for the synthesis of N-HC phenethylamines and N-HC tyramines, respectively were grown at 37 °C for 18 h in LB medium containing 50 µg/mL spectinomycin. The culture was inoculated into a fresh new LB medium containing
50 μg/mL spectinomycin. Cells were grown until OD_{600} = 0.8, at which point 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added, and continued to grow at 18 °C for a further 24 h. The cells were harvested and resuspended in M9 medium containing 2 % (w/v) glucose, 1 % (w/v) yeast extract, either 500 μM HCA (for the synthesis of N-HC phenethylamines) or 1 mM HCA (for the synthesis of HC-tyramine), 100 μg/ml antibiotic(s), and 1 mM IPTG. Fourteen HC derivatives (p-coumaric acid, m-coumaric acid, o-coumaric acid, caffeic acid, ferulic acid, cinnamic acid, 3-methoxycinnamic acid, 4-methoxycinnamic acid, 3,4-dimethoxycinnamic acid, 2,4-dimethoxyccinnamic acid, sinapic acid, and 3,4,5-trimethoxycinnamic acid) were used, individually. The cultures were incubated at 30 °C for 3 h and then extracted with ethylacetate. After drying the aqueous layer and dissolving it in dimethylsulfoxide (DMSO), the product was analyzed by HPLC. The relative N-HC phenethylamine or N-HC tyramine formation rates were calculated relative to that of the best substrate, which was denoted as 100 %.

The metabolites were analyzed using an UltiMate 3000 HPLC (Thermo Scientific, Waltham, MA, USA) equipped with a photo diode array (PDA) detector and a C18 reversed-phase column (4.60 × 250 mm, 3.5 μm particle size, Varian, Palo Alto, CA, USA). The mobile phase consisted of 0.1 % (v/v) formic acid in water and acetonitrile. The program was as follows: 20 % (v/v) acetonitrile at 0 min, 60 % at 8 min, 90 % at 12 min, 90 % at 15 min, 20 % at 15.1 min, 20 % at 20 min. The flow rate was 1 ml/min and UV absorbance was monitored at 278 and 310 nm.

Mass spectrometry (MS) was carried out as described previously [32]. The structures of N-(p-coumaroyl) phenethylamine and N-(p-coumaroyl) tyramine were determined using nuclear NMR [33]. The NMR data were as follows; N-(p-coumaroyl) phenethylamine: 1H NMR (400 MHz, DMSO-d6) δ 7.39 (d, J = 8.6 Hz, 2H), 7.34 (d, J = 15.7 Hz, 1H), 7.29 (m, 2H), 7.22 (d, J = 7.97, 2H), 7.20 (dd, J = 16.4, 7.5 Hz, 1H), 6.80 (d, J = 8.6 Hz, 2H), 6.42 (d, J = 15.7 Hz, 1H), 3.41 (m, 2H), 2.78 (t, J = 3.5 Hz, 2H); 13C NMR (100 MHz, DMSO-d6) δ 165.3, 158.8, 139.5, 138.6, 129.2, 128.6, 128.3, 126.0, 125.9, 118.6, 115.7, 40.3, 35.2.

N-(p-Coumaroyl) tyramine: 1H NMR (400 MHz, DMSO-d6) δ 8.00 (s, NH), 7.38 (m, 2H), 7.31 (d, J = 15.7 Hz, 1H), 7.00 (m, 2H), 6.79 (m, 2H), 6.68 (m, 2H), 6.40 (d, J = 15.7 Hz, 1H), 3.32 (dd, J = 7.4, 6.3 Hz, 2H), 2.64 (t, J = 7.4 Hz, 2H); 13C NMR (100 MHz, DMSO-d6) δ 165.3, 158.8, 155.6, 138.5, 129.5, 129.4, 129.1, 125.9, 118.7, 115.7, 115.1, 40.6, 34.4.

N-(p-Coumaroyl) phenethylamine and N-(p-coumaroyl) tyramine were collected and used as standards for the calculation of the production of other N-HC phenethylamines and N-HC tyramines, respectively.

**Total synthesis of N-(p-coumaroyl) phenethylamine and N-(p-coumaroyl) tyramine**

Overnight cultures of _E. coli_ strains, HTs or HPs (Table 1) were inoculated into 15 ml of fresh LB medium containing appropriate antibiotics and were cultured to OD_{600} = 1. Cells were harvested by centrifugation and cell density was adjusted to OD_{600} = 2 with 10 ml of M9 medium containing 2 % (w/v) glucose, 1 % (w/v) yeast extract, 50 μg/mL antibiotics, and 1 mM IPTG in a 100 ml flask. The cells were grown at 30 °C with shaking at 180 rpm for 24 h. To analyze product formation, cell growth was monitored by determining the absorbance at 600 nm. Culture supernatants were collected, extracted twice with an equal volume of ethyl acetate, and then dried under vacuum. The dried samples were dissolved in DMSO and analyzed using HPLC.

The mean and the standard deviation (SD) were calculated from triplicate experiments. Analysis of variance (ANOVA) was carried out using Tukey’s method, with significance at a P value of 0.01, using Microsoft Excel.

**Authors’ contributions**

JHA initiated and coordinated the project. GYS, SMY, BGK, and JHA performed the experiments and analyzed data. GYS, SMY, and JHA wrote the paper. All authors read and approved the final manuscript.

**Author details**

1. Department of Bioscience and Biotechnology, Bio/Molecular Informatics Center, Konkuk University, Seoul 143-701, Republic of Korea. 2. Department of Forest Resources, Gyeongnam National University of Science and Technology, 3 Dongjin-ro, Jinju-si, Gyeongsangnam-do 660-758, Republic of Korea.

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**Competing interests**

The authors declare that they have no competing interests.

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