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Laboratory-scale production of (*S*)-reticuline, an important intermediate of benzylisoquinoline alkaloids, using a bacterial-based method

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Benzylisoquinoline alkaloids (BIAs) are a group of plant secondary metabolites that have been identified as targets for drug discovery because of their diverse pharmaceutical activities. Well-known BIAs are relatively abundant in plants and have therefore been extensively studied. However, although unknown BIAs are also thought to have valuable activities, they are difficult to obtain because the raw materials are present at low abundance in nature. We have previously reported the fermentative production of an important intermediate (*S*)-reticuline from dopamine using *Escherichia coli*. However, the yield is typically limited. Here, we improved production efficiency by combining *in vivo* tetrahydropapaveroline production in *E. coli* with *in vitro* enzymatic synthesis of (*S*)-reticuline. Finally, 593 mg of pure (*S*)-reticuline was obtained from 1 L of the reaction mixture. Because this bacterial-based method is simple, it could be widely used for production of (*S*)-reticuline and related BIAs, thereby facilitating studies of BIAs for drug discovery.

Key words: reticuline; benzylisoquinoline alkaloid; bacterial production; drug discovery; synthetic biology

Benzylisoquinoline alkaloids (BIAs) are plant secondary metabolites, some of which have been used for medicinal applications. For example, the BIA morphine has been used for thousands of years, and its derivatives are currently prescribed as analgesic drugs for various ailments, including toothache, rheumatism, and cancer. Berberine is classified as a protoberberine alkaloid, a type of BIA, and is found in some herbal medicines. Berberine has antibacterial activity and has therefore been used as an antidiarrheal drug. Moreover, the aporphine alkaloid glaucine has been shown to have bronchodilator activity and is used as an antitussive drug. Because many known BIAs have pharmaceutical activities, unidentified or unnatural BIAs would also be expected to have various pharmaceutical activities. However, although widely used BIAs are relatively abundant in plant, many other BIAs are scarce; therefore, the physiological activities of rare BIAs are difficult to verify. Furthermore, even if the beneficial activity of a rare BIA could be discovered, it would be difficult to modify BIA to improve or alter its activity. Thus, this low abundance has hindered studies of the utility of unknown and unnatural BIAs for drug discovery, similar to other plant secondary metabolites.[1)]

Microbial production of BIAs has recently attracted attention as a new strategy for obtaining abundant amounts of BIAs.[2–7)] Genetic manipulation in bacteria is easier than that in plants, and cultivation of microbes requires less time, space, and labor compared with that of plants. Although microbial production systems are tractable, productivity may be quite low. Even tetrahydropapaveroline (THP) production systems, which have been the most successful production systems reported to date, yield only up to 287 mg/L THP.[5)]

Almost all BIAs are synthesized via the intermediate (*S*)-reticuline (Fig. 1(A)), and (*S*)-reticuline is often a target of BIA production in microbial cells and plant cells.[2–4,8–10)] Moreover, microbial BIA production systems often use (*S*)-reticuline as a starting material,[8,11)] and some BIAs have been produced from (*S*)-reticuline using enzymatic reactions[12–14)] or chemical syntheses.[15)] However, because (*S*)-reticuline does not accumulate well in plants due to its intermediate properties, obtaining sufficient amounts of (*S*)-reticuline is also difficult. Therefore, producing sufficient amounts of (*S*)-reticuline would enable the production of rare or

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Abbreviations: 3,4-DHPAA, 3,4-dihydroxy phenylacetaldehyde; 4′OMT, 3′-hydroxy-*N*-methyl-coclaurine 4′-*O*-methyltransferase; 6OMT, norcoclaurine 6-*O*-methyltransferase; BIA, benzylisoquinoline alkaloid; CID, collision-induced dissociation; Cj, *Coptis japonica*; CNMT, coclaurine *N*-methyltransferase; HPLC, high-performance liquid chromatography; IPTG, isopropyl-*β*-D-thiogalactopyranoside; MAO, monoamine oxidase; NCS, norcoclaurine synthase; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; Ps, *Papaver somniferum*; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; THP, tetrahydropapaveroline.

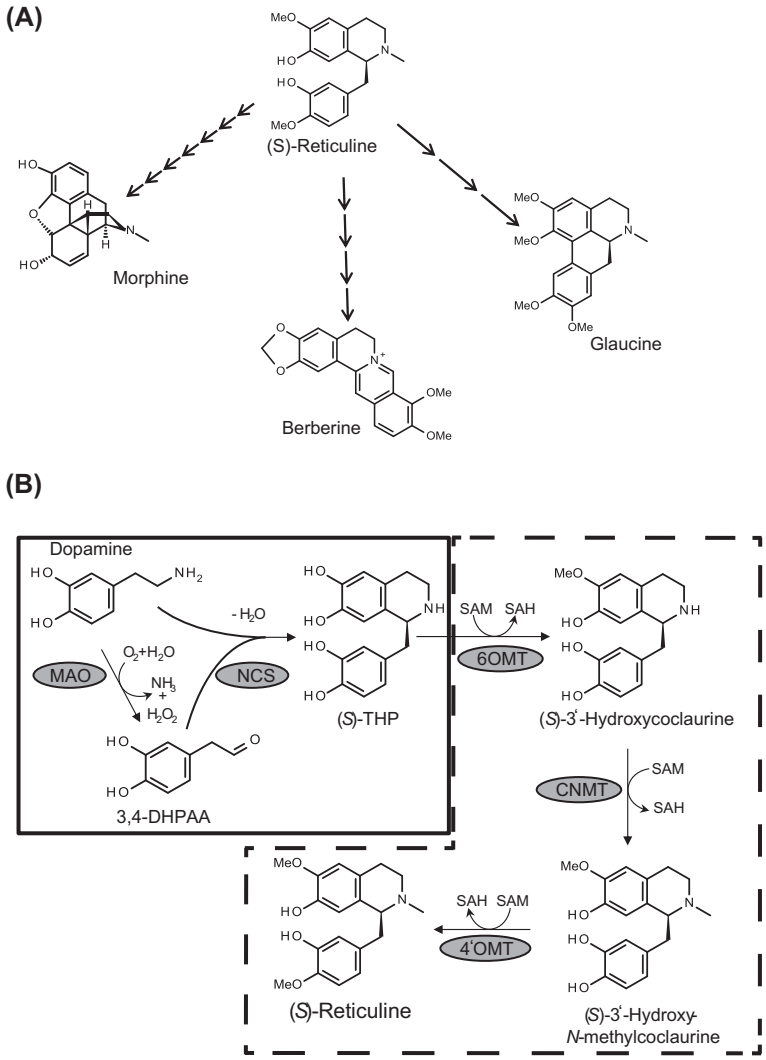

Fig. 1. The intermediate reticuline and its synthetic pathway.

Notes: (A) Well-known BIAs derived from (*S*)-reticuline. The number of arrows indicates the enzymatic reaction steps. (B) (*S*)-reticuline synthetic pathway in this study. (*S*)-THP was produced from dopamine by *in vivo E. coli* culture (surrounded by a square). (*S*)-reticuline was synthesized from (*S*)-THP by *in vitro* reaction (surrounded by dashed lines).

unnatural BIAs for assessment of their physiological activities, thereby facilitating drug discovery.

We have previously demonstrated (*S*)-reticuline production from commercially available dopamine using the *Escherichia coli* fermentation system; however, the yield in this system was limited to 54 mg/L.[16] Additionally, in our previous report, we showed that increasing the supply of dopamine to more than 1 mM does not improve (*S*)-reticuline productivity.[16]

Accordingly, in this study, we developed an improved system to produce large amounts of (*S*)-reticuline from much higher concentrations of dopamine than in our previous report. We combined microbial production of (*S*)-THP with (*S*)-reticuline synthesis by enzymatic reaction using crude extracts of *E. coli* strains expressing a methyltransferase. In this system, we succeeded to produce 677 mg/L of (*S*)-reticuline from 6.3 g dopamine hydrochloride, and after purification, 593 mg pure (*S*)-reticuline was obtained. This yield would enable the production of sufficient amounts of rare or unnatural BIAs for verification of their physiological activities by bioassays.

Materials and methods

Plasmids and E. coli *strains used in this study.* The plasmids and *E. coli* strains used in this study are listed in Supplementary Table S1. Codon usage for all genes was optimized for *E. coli* expression. The optimized sequences of norcoclaurine synthase (NCS) of *Coptis japonica*, norcoclaurine 6-*O*-methyltransferase of *Papaver somniferum* (Ps6OMT), coclaurine *N*-methyltransferase of *P. somniferum* (PsCNMT), and 3′-hydroxy-*N*-methyl-coclaurine 4′-*O*-methyltransferase 2 of *P. somniferum* (Ps4′OMT2) are shown in Supplementary Fig. S1. Those of the other genes are described in a previous report.[17] The N-terminus of NCS was deleted for *E. coli* expression.[18] Each gene was amplified by polymerase chain reaction (PCR) using the primer sets listed in Supplementary Table S2. DNA fragments, except for *Ps6OMT*, were cloned into *Nde*I-*Bam*HI sites of pET23a by In-Fusion HD or a conventional ligation method to fuse them with a T7 promoter. *Nde*I-*Xho*I sites were used for *Ps6OMT*. To generate pET23a-*NCS-MAO*, T7-*MAO* was amplified by PCR using the primers pr339 and pr379 and

subsequently cloned into the *Xho*I site of pET23a-*NCS*. *E. coli* BL21(DE3) cells were transformed with pET23a-*NCS*-*MAO*, generating the (*S*)-THP producer AN2616. To construct methyltransferase expression strains, *E. coli* BL21(DE3) cells were transformed with cognate methyltransferase expression plasmids.

(S)-THP production from dopamine. Overnight cultures of the (*S*)-THP producer AN2616 were inoculated into 50 mL Terrific Broth (12 g/L tryptone [BD Bioscience, USA], 24 g/L yeast extract [BD Bioscience], 9.4 g/L K_2HPO_4, and 2.2 g/L KH_2PO_4) containing 50 mg/L ampicillin and 0.4% glycerol in a 300-mL baffled shake flask. The cultures were grown at 25 °C. Isopropyl-*β*-D-thiogalactopyranoside (IPTG; final concentration, 1 mM) was added for induction at 12 h after inoculation. After an additional incubation for 6 h, 100 mM 2-(*N*-morpholino) ethanesulfonic acid, 10 mM ascorbate, and 100 mM dopamine were added to the culture. For efficient production of (*S*)-THP, a 50-mL sample was divided into two, and (*S*)-THP production was carried out at 29 °C.

In vitro *analysis of methyltransferases.* Overnight cultures of methyltransferase expression strains were inoculated into 50 mL Terrific Broth containing 50 mg/L ampicillin and 0.4% glycerol in a 300-mL baffled shake flask. The cultures were grown at 25 °C for 24 h. We have previously reported that IPTG induction inhibits methyltransferase activities.[17] Therefore, IPTG was not added to the medium. After cultivation, cells were harvested by centrifugation and suspended in 100 mM sodium phosphate buffer (pH 7.3) containing 10% glycerol. The crude extract containing methyltransferases was obtained by centrifugation following sonication. The protein concentration was measured using a BCA Protein Assay Kit (Pierce). The reaction mixture for measurement of (*S*)-reticuline productivity contained 4.0 mM (*S*)-THP produced from dopamine (not purified other than centrifugation), 65 μg of total protein from each methyltransferase-expressing strain, 30 mM *S*-adenosylmethionine (SAM) tosylate (neutralized by NaOH at pH 7.0; Carbosynth, UK), and 100 mM potassium phosphate (pH 7.0). The samples were incubated at 37 °C for 30 min. The reaction was stopped by addition of 2% trichloroacetate. Reticuline productivity was compared by determining the production yield for each combination. All experiments were conducted three times.

Laboratory-scale production of (S)-reticuline. THP production from 100 mM dopamine was carried out as described above. For 1 L of the (*S*)-reticuline production mixture, 333 mL of (*S*)-THP containing supernatant was required; therefore, we prepared AN2616 cultures in 14 baffled-flasks (25 mL each). The samples were harvested at 9 h after dopamine addition. Crude extracts containing each methyltransferase were prepared as described above. The crude extract contained total protein ranging from 6.42 to 7.11 g/L. The reaction mixture was comprised of 333 mL THP-containing supernatant, 100 mL of each crude extract, 30 mM SAM tosylate (pH 7.0), and 100 mM potassium phosphate (pH 7.0). Reactions were carried out at 37 °C for 8 or 9 h.

Purification of (S)-reticuline. $NaHCO_3$ and NaCl were added to the (*S*)-reticuline-containing solution until saturated. The mixture was extracted with $CHCl_3$, and the combined organic extracts were dried (Na_2SO_4), filtered, and concentrated in vacuo. The residue was purified by flash column chromatography (Kanto Chemical silica gel 60 N, spherical, neutral, 40–50 μm) to give (*S*)-reticuline (chloroform-methanol, 19:1 to 9:1). High-resolution mass-spectrometry analysis was used to detect the purified compound at $[M + H]^+ = 330.1702$, which was almost identical to the calculated value of reticuline ($C_{19}H_{24}NO_4$, 330.1705).

Detection and quantification of chemical compounds. To measure chemical compounds, samples were collected, and proteins were precipitated with 2% trichloroacetate. All compounds were separated using an Agilent high-performance liquid chromatography (HPLC) system (Agilent) as follows: column, TSKgel ODS-80Ts (4.6 × 250 mm, 5-μm particles; Tosoh); solvent system, A: 0.1% acetic acid in water, B: 0.1% acetic acid in acetonitrile; gradient modes: 90% A (0–5 min), 90–60% A (5–20 min) and 10% A (20–30 min); flow rate, 0.5 mL/min at 40 °C. The separated supernatant was analyzed by liquid chromatography mass spectrometry (LC-MS; 3200 QTRAP; Applied Biosystems, Foster City, CA, USA) using the selected ion mode (THP: $m/z = 288$; reticuline: $m/z = 330$). Compounds were identified by comparison with pure compounds with regard to their retention time and fragmentation spectrum in LC-MS/MS. The amounts of THP and reticuline were estimated from standard curves using Analyst 1.4.1 software (Applied Biosystems). The chirality of THP[5] and reticuline[4] was analyzed as previously described. Purity was assessed using HPLC, as described for LC-MS analysis, and absorbance was measured at 280 nm.

Nuclear magnetic resonance (NMR) analysis of purified (S)-reticuline. ^1H NMR and ^{13}C NMR spectra were recorded on a JEOL JNM-ECA 400 (JEOL RESONANCE Inc., Japan). ^1H and ^{13}C NMR data were analyzed as follows: δ_H (400 MHz, $CDCl_3$) 2.46 (3H, s, NCH_3), 2.55–2.60 (1H, m, H-4a), 2.71–2.74 (1H, m, H-3a), 2.77–2.80 (1H, m, H-*α*a), 2.82–2.84 (1H, m, H-4b), 3.02 (1H, dd, $J = 14.0$, 6.4 Hz, H-*α*b), 3.14–3.20 (1H, m, H-3b), 3.67 (1H, t, $J = 6.4$ Hz, H-1), 3.85 (3H, s, OCH_3), 3.86 (3H, s, OCH_3), 6.41 (1H, s, H-5), 6.54 (1H, s, H-8), 6.60 (1H, dd, $J = 8.4$, 2.4 Hz, H-6′), 6.74 (1H, d, $J = 8.4$ Hz, H-5′), 6.78 (1H, d, $J = 2.4$ Hz, H-2′); δ_C (100 MHz, $CDCl_3$) 25.0 (C-4), 41.0 (C-*α*), 42.4 (NCH_3), 46.7 (C-3), 55.9 (OCH_3), 60.0 (OCH_3), 64.6 (C-1), 110.6 (C-5′), 110.7 (C-5), 113.9 (C-8), 115.8 (C-2′), 121.0 (C-6′), 125.2 (4a), 130.2 (8a), 133.2 (C-1′), 143.5 (C-7), 145.2, 145.4, 145.5 (C-3′, C-4′, C-6).

Results

THP production

To produce (*S*)-reticuline from dopamine, a five-step reaction was required (Fig. 1(B)). Dopamine was converted to 3,4-dihydroxy phenylacetaldehyde (3,4-DHPAA) by monoamine oxidase (MAO). The S-form specific enzyme NCS catalyzed the coupling reaction with 3,4-DHPAA and dopamine, converting dopamine to (*S*)-THP. (*S*)-reticuline was then produced after three methylations of (*S*)-THP by three methyltransferases (6OMT, CNMT, and 4′OMT). We have previously demonstrated that the *E. coli* fermentation system has the capacity for high THP production by optimizing culture conditions.[5)] Therefore, we attempted (*S*)-THP production from dopamine using an *in vivo* (*S*)-THP production system. The MAO and NCS expression strain AN2616 was cultured in 50 mL medium containing various concentrations of dopamine in a baffled-flask. When up to 100 mM dopamine was added, THP productivity was increased; however, when 200 mM dopamine was added to the culture, the yield was almost the same as that in cultures with addition of 100 mM dopamine (Fig. 2(A)). Thus, we decided to use 100 mM dopamine for THP production. Because the MAO reaction requires molecular oxygen, we attempted to produce THP in smaller culture volumes to increase the air supply. As a result, the yield was slightly improved in 10- and 25-mL cultures (Fig. 2(B)). Because 10-mL cultures showed the same THP production level as 25-mL cultures, we decided to conduct (*S*)-THP production in 25-mL cultures. From this reaction, 12.1 ± 1.6 mM THP was produced, representing a conversion efficiency of 24.2% ± 3.1%. AN2616 expressed NCS, which is an S-form specific enzyme; however, a small amount of the R-form of THP was detected in the culture medium (Fig. 2(C); roughly calculated to be about 25% of the total (*R*, *S*)-THP), indicating that a weak spontaneous Pictet-Spenglar reaction accompanied NCS dependent (*S*)-THP synthesis.

*Enzyme selection for (*S*)-reticuline production*

In chemical reactions, methylation of a single hydroxy group is generally complicated because other groups must be protected to avoid undesirable methylation. However, the use of enzymes does not necessitate complicated manipulations, such as protection and deprotection, due to the high regiospecificity of enzymes. We have previously reported that an *in vitro* system using cell-free extracts of *E. coli* expressing

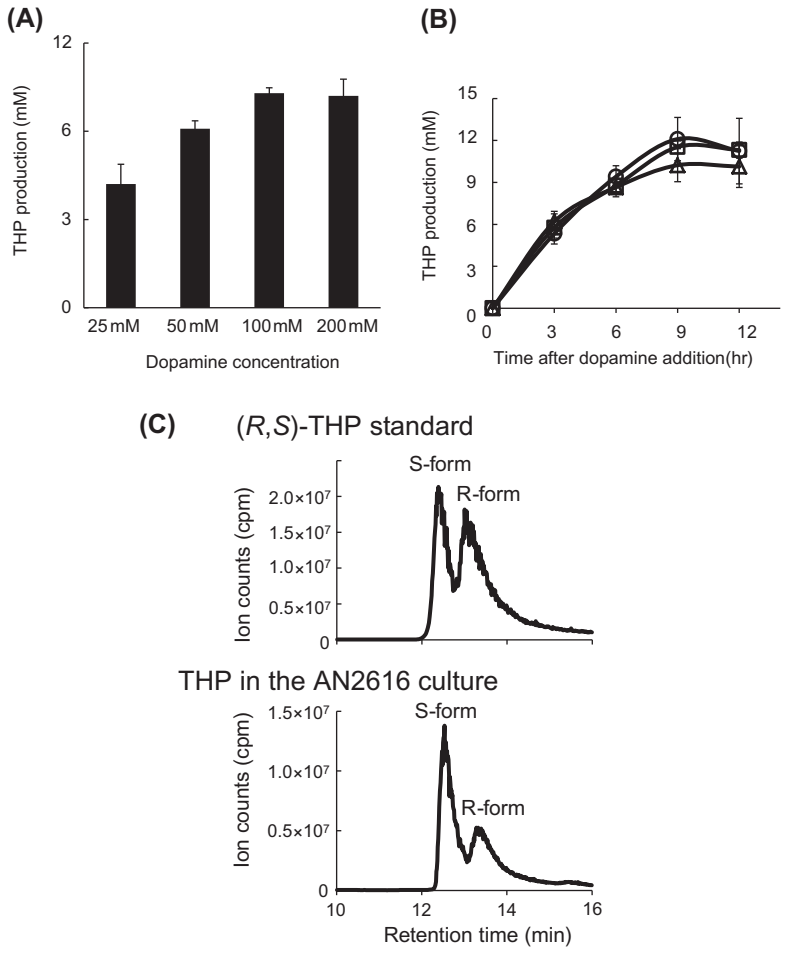

Fig. 2. THP production by an *in vivo* production system.

Notes: (A) THP was produced from different concentrations of dopamine in the 50-mL culture. Samples were harvested at 12 h after dopamine addition. (B) THP was produced in different culture volumes of 10 mL (squares), 25 mL (circles), and 50 mL (triangles). Time zero indicates the timing of dopamine addition. (C) The chirality of the produced THP was analyzed by LC-MS (*m/z* = 288) using a chiral column. The error bar indicates the standard deviation of three independent experiments.

Table 1. 6OMT and CNMT selection by *in vitro* reaction.

6OMT	CNMT	Reticuline productivity[a] (relative rate %)
Cj	Cj	100 ± 7
Cj	Ps	63 ± 5
Ps	Cj	105 ± 4
Ps	Ps	116 ± 5

[a]The productivity in Cj6OMT and CjCNMT was set as 100. Values represent the mean ± SEM from three independent experiments.

(*S*)-reticuline synthetic enzymes exhibits higher productivity than an *in vivo* fermentation system.[2)] Therefore, we used an *in vitro* system with crude extracts of *E. coli* expressing three methyltransferases for (*S*)-reticuline production from (*S*)-THP.

As laboratory stocks, we had two orthologs of 6OMTs, CNMTs, and 4′OMTs, which originated from *C. japonica* (Cj) and *P. somniferum* (Ps). According to our preliminary experiments, 4′OMT of Ps (Ps4′OMT2) did not exhibit significant activity in our *E. coli* system. To select the best combination of 6OMTs and CNMTs, we compared (*S*)-reticuline productivity from THP synthesized from dopamine (Table 1). From this analysis, we found that the highest productivity could be observed when Ps6OMT and PsCNMT were used for (*S*)-reticuline production. Notably, the activities in this experiment were reflected by expression levels and enzymatic activities. Thus, we used Ps6OMT, PsCNMT, and Cj4′OMT for (*S*)-reticuline synthesis from (*S*)-THP.

*Laboratory-scale production of (*S*)-reticuline*

Next, we carried out laboratory-scale production of (*S*)-reticuline as described above. Using centrifugation, we collected THP-containing supernatants from AN2616 cells cultured in medium containing 100 mM dopamine. THP was produced at a concentration of 3.42 ± 0.08 g/L (11.9 ± 0.3 mM, three independent experiments). The crude extracts containing 6OMT, CNMT, and 4′OMT were individually harvested from cognate strains and mixed with the THP solution (final: 4.0 mM THP). A 1-L reaction mixture was incubated at 37 °C for 8 or 9 h. The feature of the collision-induced dissociation (CID) of the products was identical with that of the (*R*,*S*)-reticuline standard (Fig. 3(A)). Although the R-form of THP was present in low abundance in the THP-containing supernatant, only the S-form of reticuline was obtained from this *in vitro* (*S*)-reticuline production system (Fig. 3(B)), suggesting that the S-form preference of methyltransferases inhibited (*R*)-reticuline formation, similar to the results of a previous report.[17)] Finally, we found that 677 ± 43 mg/L (2.06 ± 0.13 mM) of (*S*)-reticuline was produced by

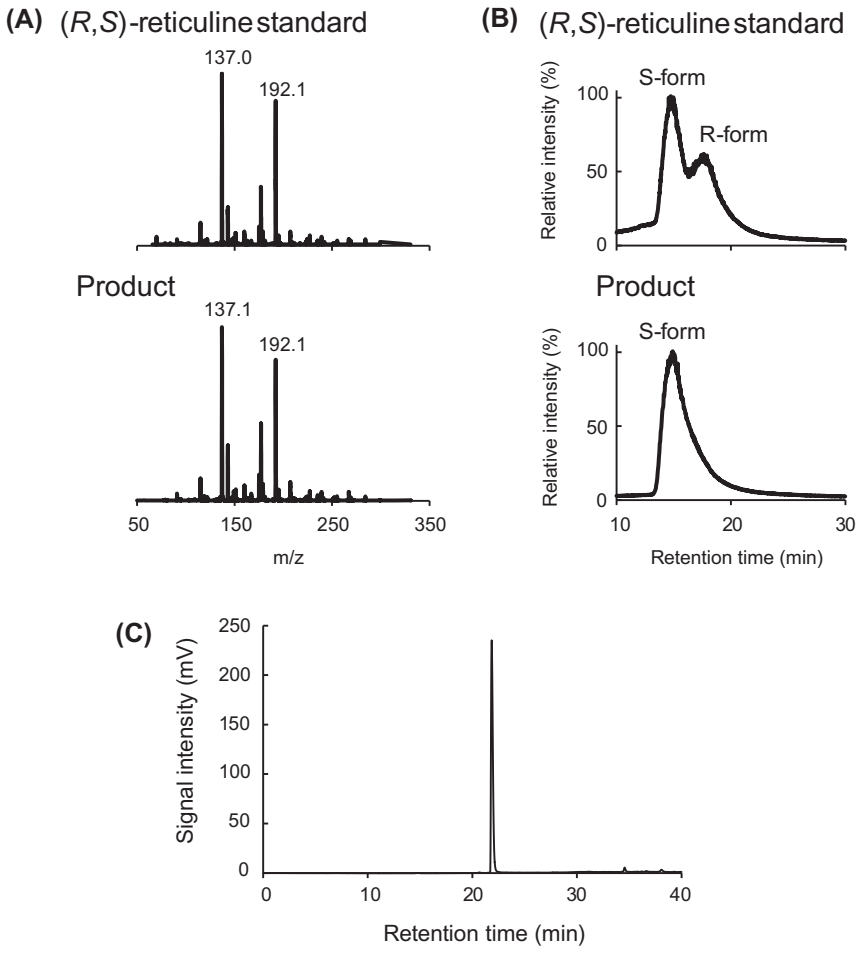

Fig. 3. Analysis of produced reticuline.

Notes: (A) Comparison of CID patterns between an (*R*,*S*)-reticuline standard and the product. (B) The chirality of the produced reticuline was analyzed by LC-MS (*m/z* = 330) using a chiral column. (C) The purity of (*S*)-reticuline after purification was analyzed by HPLC.

our system of combined microbial THP production with *in vitro* (*S*)-reticuline synthesis.

*Purification and verification of (*S*)-reticuline*

We purified (*S*)-reticuline from the reaction mixture by chloroform extraction followed by flash column chromatography using silica gel. The results showed that 593 ± 27 mg (*S*)-reticuline could be purified from a 1-L reaction mixture. The solid was colorless (Supplementary Fig. S2). The purity was more than 96% according to HPLC analysis (Fig. 3(C)), and the purification efficiency from the (*S*)-reticuline synthesized solution reached 87.5%. Furthermore, NMR analysis clearly confirmed that the purified compound was reticuline with high purity (Supplementary Fig. S3, see Materials and methods).

Discussion

In this study, we succeeded in producing approximately 600 mg of purified (*S*)-reticuline in a 1-L reaction mixture. This amount was sufficient to synthesize other BIAs via chemical synthesis or microbial production. Furthermore, other BIAs produced from the purified (*S*)-reticuline could be obtained at a sufficient level for assessment of their physiological activities. This high productivity was accomplished without special equipment such as a jar-fermenter or complicated reaction processes. The minimum time for the production was as little as 3 days from the preculture of an (*S*)-THP producer. Furthermore, the required compounds, such as dopamine, SAM tosylate, and medium, were readily available compared with expensive substrates, including (*S*)-THP. Thus, this method is simple, rapid, and cost-effective and can be performed in laboratories whose specialties are not bacteriology or plant biology.

The fermentative production of (*S*)-reticuline from dopamine was previously shown to be limited to 54 mg/L[16]; however, using the current system, we achieved an improvement of more than 12-fold (677 mg/L). In the *in vitro* reaction for (*S*)-reticuline synthesis, we could supply high amounts of SAM; therefore, the reactions used in this study were more efficient reaction than those in the *in vivo* fermentation system, in which SAM productivity may be limited.

The purification method developed in this study was very simple compared with methods for extraction from plants harboring various BIAs. Because our method was based on bacterial materials, other BIAs possessing similar chemical properties were not found in the (*S*)-reticuline-containing sample. In addition, the purification efficiency and purity were also high, indicating that bacteria-based methods are powerful tools for production of plant secondary metabolites.

Berberine is relatively abundant in nature, and its physiological activities have been extensively studied.[19] In addition, berberine derivatives synthesized by simple chemical modification have been also studied and have been shown to have stronger or novel physiological activities.[20,21] The availability of large amounts of berberine may have facilitated these studies. In contrast, although some (*S*)-reticuline derivative BIAs have been produced using engineered microbes or chemical synthesis, the low productivity of (*S*)-reticuline is one of the rate-limiting steps for the production of BIAs.[3,8,22] Thus, the high (*S*)-reticuline production achieved by the system constructed herein would enable the production of abundant amounts of rare or unnatural BIAs and may facilitate studies of BIAs as novel drugs.

Author contributions

A.N. and H.M. conceived and designed all experiments. E.M., A.N., and Y.T. performed all experiments. T. Koyanagi, T. Katayama, and K.Y. discussed the results. H.K. and F.S. supervised the project. A.N., H.M., T. Katayama, and F.S. wrote the manuscript. All authors reviewed the manuscript.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Supplemental material

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References

[1] Li JW, Vederas JC. Drug discovery and natural products: end of an era or an endless frontier? Science. 2009;325:161–165.
[2] Minami H, Kim JS, Ikezawa N, et al. Microbial production of plant benzylisoquinoline alkaloids. Proc Natl Acad Sci U S A. 2008;105:7393–7398.
[3] Hawkins KM, Smolke CD. Production of benzylisoquinoline alkaloids in *Saccharomyces cerevisiae*. Nat Chem Biol. 2008;4:564–573.
[4] Nakagawa A, Minami H, Kim JS, et al. A bacterial platform for fermentative production of plant alkaloids. Nat Commun. 2011;2:326.
[5] Nakagawa A, Matsuzaki C, Matsumura E, et al. (*R,S*)-tetrahydropapaveroline production by stepwise fermentation using engineered *Escherichia coli*. Sci Rep. 2014;4:6695.
[6] Fossati E, Ekins A, Narcross L, et al. Reconstitution of a 10-gene pathway for synthesis of the plant alkaloid dihydrosanguinarine in *Saccharomyces cerevisiae*. Nat Commun. 2014;5:3283.
[7] Galanie S, Thodey K, Trenchard IJ, et al. Complete biosynthesis of opioids in yeast. Science. 2015;349:1095–1100.
[8] Fossati E, Narcross L, Ekins A, et al. Synthesis of morphinan alkaloids in *Saccharomyces cerevisiae*. PLoS One. 2015;10:e0124459.

[9] Trenchard IJ, Siddiqui MS, Thodey K, et al. De novo production of the key branch point benzylisoquinoline alkaloid reticuline in yeast. Metab Eng. 2015;31:74–83.

[10] Fujii N, Inui T, Iwasa K, et al. Knockdown of berberine bridge enzyme by RNAi accumulates (*S*)-reticuline and activates a silent pathway in cultured California poppy cells. Transgenic Res. 2007;16:363–375.

[11] Hori K, Okano S, Sato F. Efficient microbial production of stylopine using a *Pichia pastoris* expression system. Sci Rep. 2016;6:22201.

[12] Zenk MH, Gerardy R, Stadler R. Phenol oxidative coupling of benzylisoquinoline alkaloids is catalysed by regio- and stereo-selective cytochrome P-450 linked plant enzymes: salutaridine and berbamunine. J Chem Soc Chem Commun. 1989;22:1725–1727.

[13] Frenzel T, Beale JM, Kobayashi M, et al. Stereochemistry of enzymic formation of the berberine bridge in protoberberine alkaloids. J Am Chem Soc. 1988;110:7878–7880.

[14] Schrittwieser JH, Resch V, Wallner S, et al. Biocatalytic organic synthesis of optically pure (*S*)-scoulerine and berbine and benzylisoquinoline alkaloids. J Org Chem. 2011;76:6703–6714.

[15] Vanderlaan DG, Schwartz MA. Synthesis and oxidative coupling of (±)-3-oxoreticuline. J Org Chem. 1985;50:743–747.

[16] Kim JS, Nakagawa A, Yamazaki Y, et al. Improvement of reticuline productivity from dopamine by using engineered *Escherichia coli*. Biosci Biotechnol Biochem. 2013;77:2166–2168.

[17] Nakagawa A, Matsumura E, Koyanagi T, et al. Total biosynthesis of opiates by stepwise fermentation using engineered *Escherichia coli*. Nat Commun. 2016;7:10390.

[18] Minami H, Dubouzet E, Iwasa K, et al. Functional analysis of norcoclaurine synthase in *Coptis japonica*. J Biol Chem. 2007;282:6274–6282.

[19] Kumar A, Ekavali K, Chopra K, et al. Current knowledge and pharmacological profile of berberine: an update. Eur J Pharmacol. 2015;761:288–297.

[20] Ikekawa T, Ikeda Y. Antitumor activity of 13-methyl-berberrubine derivatives. J Pharmacobiodyn. 1982;5:469–474.

[21] Lee D-U, Kang YJ, Park MK, et al. Effects of 13-alkyl-substituted berberine alkaloids on the expression of COX-II, TNF-α, iNOS, and IL-12 production in LPS-stimulated macrophages. Life Sci. 2003;73:1401–1412.

[22] Galanie S, Smolke CD. Optimization of yeast-based production of medicinal protoberberine alkaloids. Microb Cell Fact. 2015;14:144.