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Pterin-Dependent Mono-oxidation for the Microbial Synthesis of a Modified Monoterpene Indole Alkaloid

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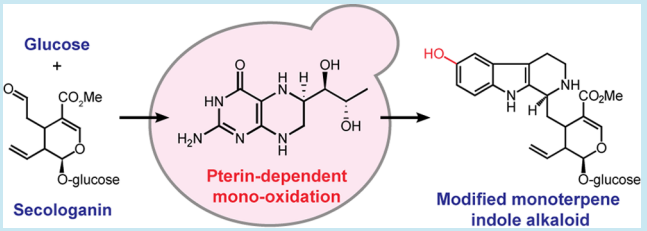
Supporting Information

ABSTRACT: Monoterpene indole alkaloids (MIAs) have important therapeutic value, including as anticancer and antimalarial agents. Because of their chemical complexity, therapeutic MIAs, or advanced intermediates thereof, are often isolated from the native plants. The microbial synthesis of MIAs would allow for the rapid and scalable production of complex MIAs and MIA analogues for therapeutic use. Here, we produce the modified MIA hydroxystrictosidine from glucose and the monoterpene secologanin *via* a pterin-dependent mono-oxidation strategy. Specifically, we engineered the yeast *Saccharomyces cerevisiae* for the high-level synthesis of tetrahydrobiopterin to mono-oxidize tryptophan to 5-hydroxytryptophan, which, after decarboxylation to serotonin, is coupled to exogenously fed secologanin to produce 10-hydroxystrictosidine in an eight-enzyme pathway. We selected hydroxystrictosidine as our synthetic target because hydroxylation at the 10′ position of the alkaloid core strictosidine provides a chemical handle for the future chemical semisynthesis of therapeutics. We show the generality of the pterin-dependent mono-oxidation strategy for alkaloid synthesis by hydroxylating tyrosine to L-DOPA—a key intermediate in benzylisoquinoline alkaloid (BIA) biosynthesis—and, thereafter, further converting it to dopamine. Together, these results present the first microbial synthesis of a modified alkaloid, the first production of tetrahydrobiopterin in yeast, and the first use of a pterin-dependent mono-oxidation strategy for the synthesis of L-DOPA. This work opens the door to the scalable production of MIAs as well as the production of modified MIAs to serve as late intermediates in the semisynthesis of known and novel therapeutics. Further, the microbial strains in this work can be used as plant pathway discovery tools to elucidate known MIA biosynthetic pathways or to identify pathways leading to novel MIAs.



KEYWORDS: *alkaloids, yeast, tetrahydrobiopterin, biogenic amines, biosynthesis*

Alkaloids are the largest group of nitrogen-containing secondary metabolites, with more than 20 000 structures,[1] are present in roughly 20% of plant species,[2] and are important because of their medicinal use.[3] Of particular importance are monoterpene indole alkaloids (MIAs), which include anticancer agents such as camptothecin (**1**) and vinblastine (**2**), antimalarial agents such as quinine, and antiarrhythmic agents such as ajmalicine. Another important alkaloid family is benzylisoquinoline alkaloids (BIAs), which include the antibiotic berberine (**3**) and the analgesic morphine (**4**).[4] Due to their chemical complexity, alkaloids often require multistep chemical syntheses, which, coupled with the necessity of enantiopure material, make them a challenging synthetic target.[5−8] Therefore, medically important alkaloids, or advanced intermediates thereof, are frequently isolated directly from the native plants.[5,7,9] Although effective, isolation of alkaloids from plants is often limited by their low accumulation in plant tissue and their difficult separation from other natural products, thus resulting in the high cost of alkaloid-derived pharmaceuticals, especially those based on MIAs.[5,7] *Via* plant breeding and, more recently, plant metabolic engineering, the production of BIAs and MIAs *in planta* has been increased.[10−13] Further, plants have been engineered to produce halogenated MIAs, with potentially higher bioactivity, which can serve as late intermediates in the semisynthesis of other alkaloids.[14−16] Nevertheless, limited understanding of plant secondary metabolite regulation and slow plant growth rate cloud the future of engineering plants to overproduce alkaloids.[2,5,7] These limitations result in the underrepresentation of alkaloid-derived compounds in pharmaceutical drug screenings.[17] The synthesis of plant alkaloids in microbes would enable the rapid and scalable production of known alkaloids and open the door to the biosynthesis of novel alkaloids using engineered enzymes or combinatorial enzyme assembly. Plant alkaloid production in microbes has the advantages of short doubling time, rapid extraction of the alkaloid from the culture medium, easier isolation of the desired alkaloid due to the absence of similar natural products, and a lack of endogenous pathway regulation, which allows for deregulation of alkaloid biosynthesis.

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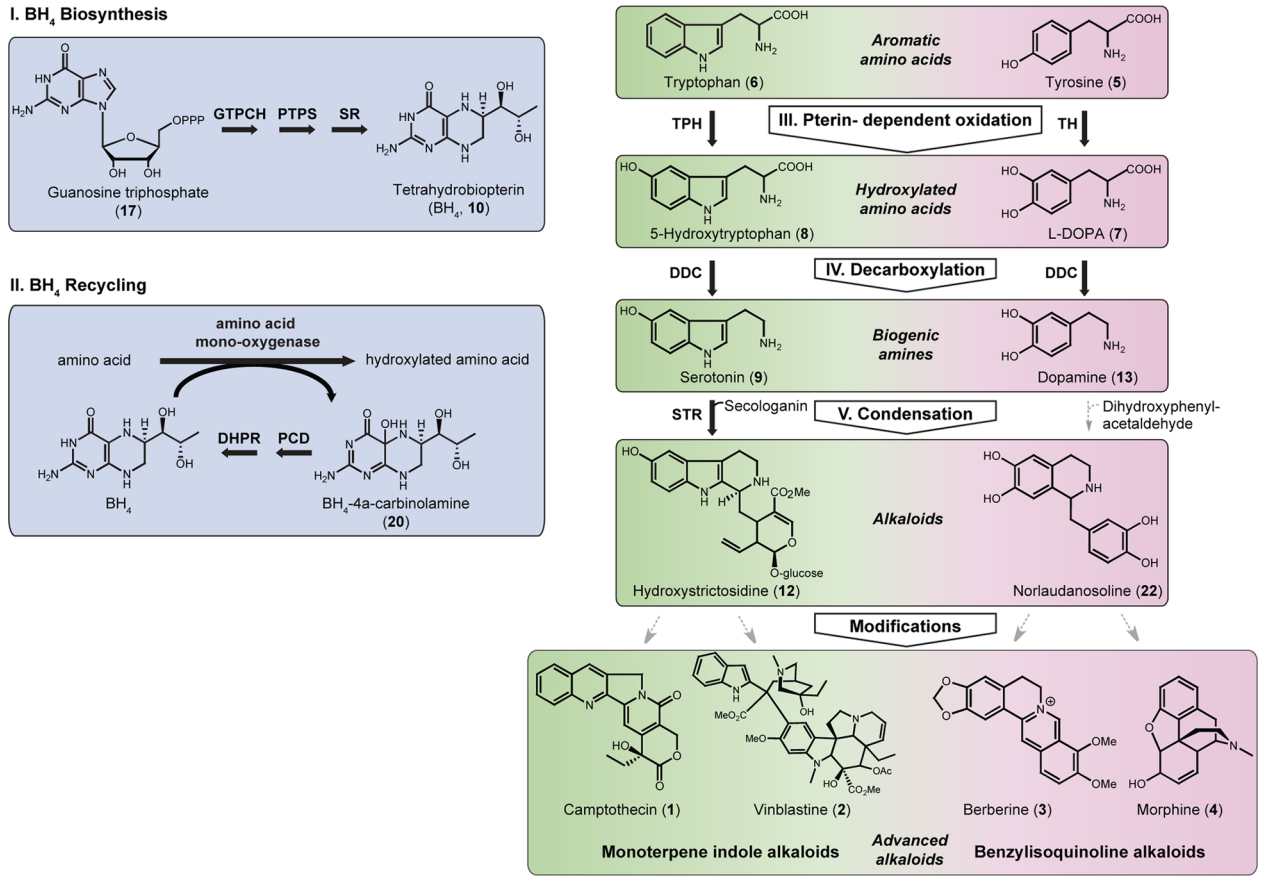

Figure 1. Pterin-dependent microbial synthesis of monoterpene indole alkaloids (MIAs) in *Saccharomyces cerevisiae*. General schematic of MIA and benzylisoquinoline alkaloid microbial synthesis via pterin-dependent mono-oxidation. The engineered strains of *S. cerevisiae* presented in this work contain one or more of the following: (I) the BH_4 biosynthetic pathway, (II) the BH_4 recycling pathway, (III) a pterin-dependent mono-oxygenase, (IV) a decarboxylase, or (V) a Pictet−Spenglerase. Gray arrows represent future potential of the system. GTPCH, GTP cyclohydrolase; PTPS, pyruvoyl tetrahydropterin synthase; SR, sepiapterin reductase; PCD, pterin-4a-carbinolamine dehydratase; DHPR, dihydropteridine reductase; TPH, tryptophan hydroxylase; TH, tyrosine hydroxylase; DDC, aromatic-L-amino-acid decarboxylase; STR, strictosidine synthase.

Many alkaloids are obtained *via* the hydroxylation and decarboxylation of amino acids.[18] Specifically, BIAs are derived from tyrosine (**5**) and MIAs are derived from tryptophan (**6**). In the last 10 years, full elucidation of many BIA biosynthetic pathways,[4] in conjunction with advances in synthetic biology, have enabled the reconstruction of BIA pathways in both *Escherichia coli* and *Saccharomyces cerevisiae*.[19−25] Although MIA biosynthetic pathways have been extensively engineered *in planta*,[14,15] the engineering of MIA alkaloids in microbes has been limited. A major problem in engineering microbes for the synthesis of plant alkaloids is the amino acid hydroxylation step. Tyrosinase, the most common enzyme used to hydroxylate tyrosine, not only oxidizes tyrosine into L-3,4-dihydroxyphenylalanine (L-DOPA, **7**), but its *o*-diphenolase activity also results in further oxidation of L-DOPA to L-dopaquinone, a melanin precursor,[26−28] thus reducing the availability of L-DOPA for alkaloid production. Nevertheless, tyrosinase has been used to produce the BIA reticuline from glycerol.[21,25] Recently, a P450 enzyme from beet was engineered for reduced *o*-diphenolase activity to increase the specificity of tyrosine hydroxylation to L-DOPA.[29] There is no equivalent to tyrosinase for tryptophan hydroxylation. To circumvent this problem, microbial synthesis of 5-hydroxytryptophan (**8**) has been achieved by indole hydroxylation followed by coupling to serine[30] or using an engineered phenylalanine hydroxylase with changed substrate specificity.[31] Hydroxytrytophan has not been converted to serotonin (**9**) or to MIAs microbially from glucose. Specific mono-oxygenases for tyrosine and tryptophan exist in higher eukaryotes; however, they require the pterin cofactor tetrahydrobiopterin (BH_4, **10**), which is not present in *E. coli* or *S. cerevisiae*.

We hypothesized that a pterin-dependent oxidation strategy to specifically mono-oxidize tyrosine or tryptophan would provide an alternative route for MIA and BIA biosynthesis (Figure 1). The difficulty in this strategy is that neither *E. coli* nor *S. cerevisiae* endogenously produces BH_4, which is necessary for the activity of mono-oxygenases found in higher eukaryotes.[32,33] Although BH_4 has been previously produced in *E. coli*,[34] it was not coupled to amino acid mono-oxidation. The endogenous *E. coli* BH_4 analogue, tetrahydromonapterin (MH_4), has been promiscuously used as a cofactor for BH_4-dependent mono-oxygenases in the production of hydroxytyrosol[35] and 5-hydroxytryptophan.[31] However, MH_4 does not have the correct composition or stereochemistry when compared to BH_4 (Supporting Information (SI) Figure 1), which is likely to be accepted with higher efficiency by amino acid mono-oxygenases from higher eukaryotes,[32,36] such as those in this study. To establish a microbial platform for the synthesis of plant alkaloids *via* the pterin-dependent mono-oxidation strategy, we engineered *S. cerevisiae* for BH_4 production. We selected *S. cerevisiae* as the microbial host because yeast, like plants, is eukaryotic. A yeast platform for

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amino acid mono-oxidation will facilitate the synthesis of complex plant alkaloids, as expression of downstream alkaloid pathway enzymes are thought to be mainly transmembrane cytochrome P450s, which are difficult to functionally express in bacteria such as *E. coli*[20,37] without protein engineering.[38−40] In addition, *S. cerevisiae*'s robustness, tolerance to industrial conditions, including low pH and high sugar concentrations, and insusceptibility to phage infection make it the ideal host for chemical production.[41−43]

Here, we report the use of a pterin-dependent mono-oxidation strategy for the microbial synthesis of the biogenic amines dopamine and serotonin and the leveraging of serotonin to produce a modified MIA. Specifically, we engineered BH_4-producing yeast to mono-oxidize tryptophan to 5-hydroxy-tryptophan, which, after decarboxylation to serotonin, is condensed with the monoterpene secologanin (**11**) to produce the modified MIA hydroxystrictosidine (**12**). First, we combinatorially screened BH_4 biosynthetic enzymes to identify the enzyme assembly leading to the highest BH_4 production. Next, we introduced a BH_4 recycling pathway to guarantee supply of BH_4 to the amino acid mono-oxygenases. Then, we showed that pterin-dependent oxidation of tryptophan followed by decarboxylation results in serotonin, a key MIA intermediate. Finally, we introduced the MIA biosynthetic pathway to ultimately produce hydroxystrictosidine from glucose and secologanin. We show the generality of the pterin-dependent mono-oxidation strategy for the microbial synthesis of alkaloids by using a tyrosine mono-oxygenase to convert tyrosine into L-DOPA, which is subsequently decarboxylated to dopamine (**13**), a key BIA intermediate. This is the first production of BH_4 in *S. cerevisiae* and the first microbial synthesis of a modified MIA. Further, this is the first time that *S. cerevisiae* has been engineered to produce the key BIA biogenic amine, dopamine, and the key MIA biogenic amine, serotonin, from glucose *via* pterin-dependent mono-oxidation. The microbial strains presented in this work open the door to the scalable production of MIAs, as well as the production of modified MIAs to serve as late intermediates in the semisynthesis of known and novel therapeutics. Further, the microbial strains in this work can be used as plant pathway discovery tools to elucidate known MIA biosynthetic pathways or to identify pathways leading to novel MIAs.

RESULTS

Target Choice: Hydroxystrictosidine. While the natural branch point in MIA biosynthesis is strictosidine,[44] we pursued instead the biosynthesis of 10-hydroxystrictosidine, an MIA that can be produced by *Camptotheca acuminata*,[45] the major producer of the anticancer agent camptothecin. We were interested in the biosynthesis of 10′ functionalized strictosidine as it provides a chemical handle for the rapid derivatization of strictosidine-derived MIAs. 10-hydroxystrictosidine is synthesized *via* the condensation of 5-hydroxytryptamine (serotonin) and secologanin, rather than tryptamine and secologanin as in the case of strictosidine. Modifications at the 5′ position of tryptophan have been shown to be processed by MIA enzymes in *Catharanthus roseus* to produce 10′ modified ajmalicine, serpentine, and tabersonine.[46] In this spirit, 10-hydroxystrictosidine may enable the biosynthesis of modified MIAs, such as 10-hydroxycamptothecin (**14**), which has higher anticancer activity than camptothecin[18] (Figure 2). Modified MIAs such as 10-hydroxycamptothecin can serve as better semisynthesis intermediates than camptothecin for the chemical synthesis of more water-soluable derivatives,[18] such as the colon anticancer drug irinotecan (**15**) and the ovarian and lung cancer drug topotecan (**16**). More generally, modified MIAs can serve as synthons for the semisynthesis of novel complex alkaloids with potential therapeutic activities.

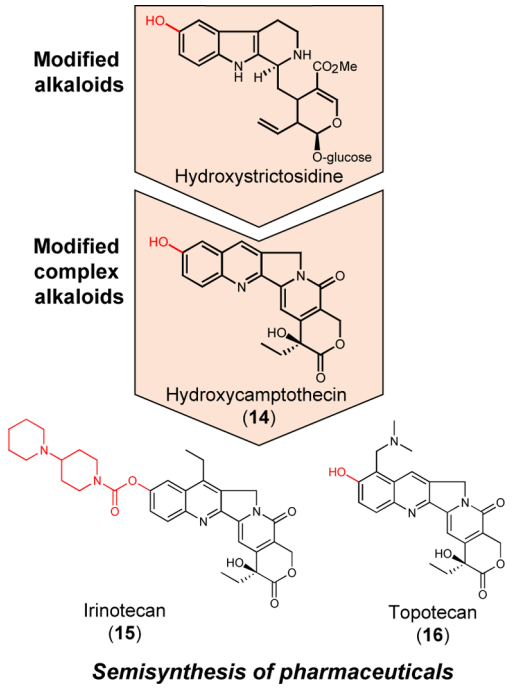

Figure 2. Modified MIAs for the semisynthesis of pharmaceuticals. Modified alkaloids, such as hydroxystrictosidine, have the potential to be biosynthetically converted to advanced alkaloids, such as hydroxycamptothecin, *via* the MIA biosynthetic pathway. These alkaloids can be used as functionalized starting materials for the semisynthesis of pharmaceuticals. In particular, hydroxycamptothecin, which is the 10′ hydroxylated version of camptothecin, could enable rapid access to the anticancer drugs topotecan and irinotecan.

Microbial Synthesis of Tetrahydrobiopterin in *S. cerevisiae*. *S. cerevisiae* does not produce BH_4, but guanosine triphosphate (GTP, **17**) can be rerouted to produce BH_4 through the intermediates dihydroneopterin triphosphate (**18**) and pyruvoyl tetrahydropterin (**19**) using three enzymes: GTP cyclohydrolase I (GTPCH), pyruvoyl tetrahydropterin synthase (PTPS), and sepiapterin reductase (SR) (Figure 3a). Given that BH_4 oxidizes to dihydrobiopterin and, subsequently, to biopterin in water,[47] we screened for the presence of biopterin in the medium. GTPCH is the first committed step in BH_4 biosynthesis.[48] *S. cerevisiae* has an endogenous GTPCH as part of the folate biosynthetic pathway and thus requires only expression of heterologous PTPS and SR to produce BH_4. *Mortierella alpina* is the only fungus shown to carry the full BH_4 biosynthetic pathway from GTP,[49] and, given that *S. cerevisiae* is also a fungus, *M. alpina* enzymes may be efficiently expressed in this organism. Overexpression of *M. alpina* PTPS and SR in *S. cerevisiae* produced 0.74 mg/L of BH_4, measured as biopterin (Figure 3b, SI Figure 2). Concerned that this would be an insufficient amount of BH_4, even for use as a cofactor only, we set out to determine if BH_4 levels have a limiting effect on alkaloid production. We exogenously fed BH_4 to yeast cells expressing only a pterin-dependent tyrosine mono-oxygenase to convert tyrosine into L-DOPA. No L-DOPA was seen when feeding 0−50 mg/L of BH_4, suggesting that BH_4 oxidizes to

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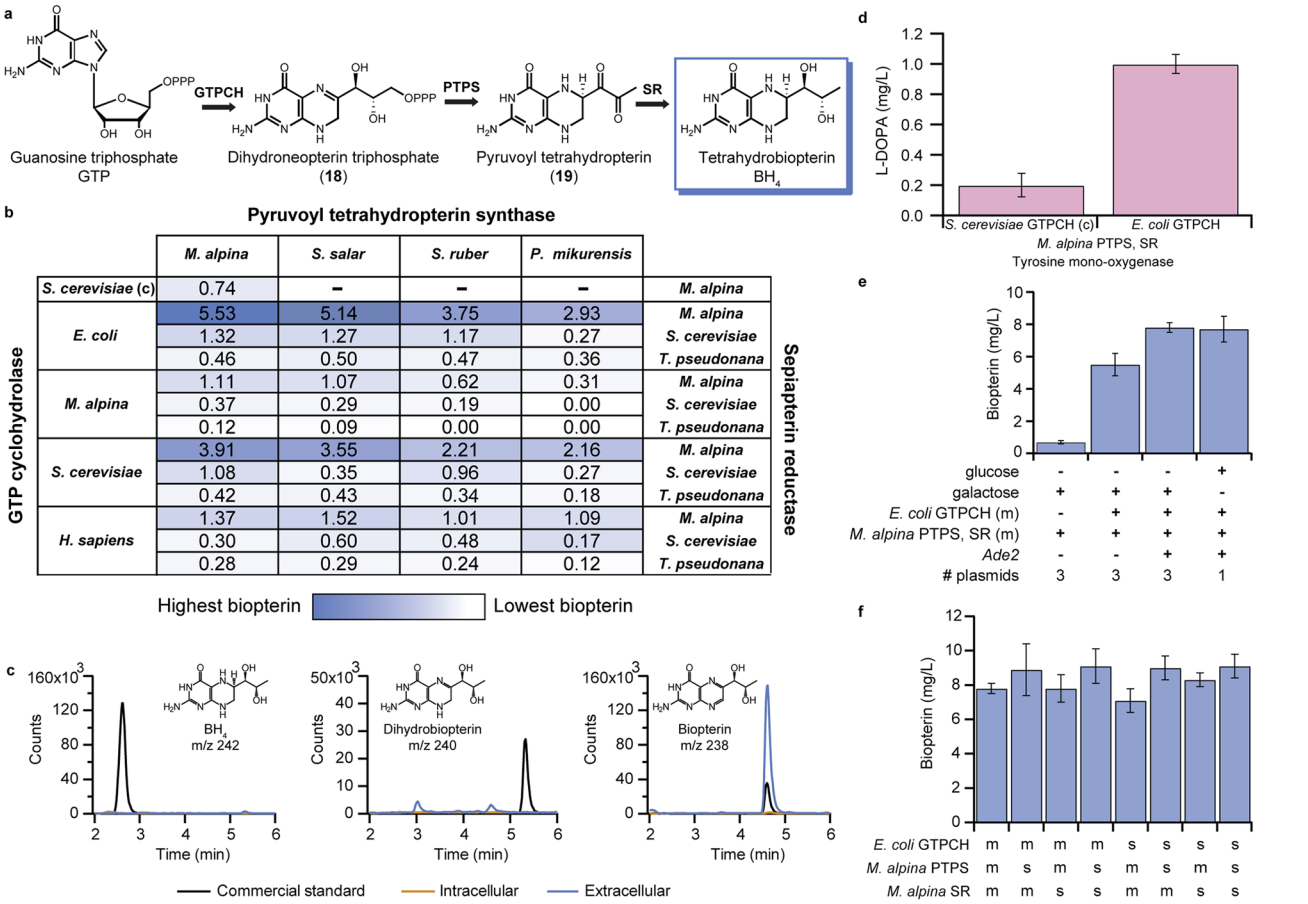

Figure 3. Microbial synthesis of tetrahydrobiopterin (BH_4) in *S. cerevisiae*. (a) BH_4 biosynthetic pathway from guanosine triphosphate (GTP), which is endogenously made by *S. cerevisiae*. (b) Heat map of biopterin titers (mg/L), as a proxy for BH_4 titers, from the 49 BH_4-producing yeast strains. Except for *S. cerevisiae* chromosomal (c), where the chromosomal copy of *S. cerevisiae* GTPCH was used, each enzyme was expressed from a multicopy plasmid from an inducible galactose promoter (P_{GAL1}). Biopterin was quantified using liquid chromatography−mass spectrometry (LC−MS). Biopterin titers reported as (-) were not determined. Biopterin titers reported as 0.00 were either too low to quantify or undetectable. A control strain expressing green fluorescent protein in a three-plasmid system showed no biopterin production. The experiments were run in triplicate and shown are the means. Standard deviations can be found in SI Figure 2. (c) LC−MS traces (extracted ion chromatograms) of BH_4 (m/z 242) and oxidation products dihydrobiopterin (m/z 240) and biopterin (m/z 238) found intracellularly and in the production medium. Standard retention times: BH_4 = 2.6 min, dihydrobiopterin = 5.3 min, biopterin = 4.6 min. Only biopterin was observed in the production medium. Full windows of the spectra can be found in SI Figure 2. (d) Pterin-dependent mono-oxidation of tyrosine to L-DOPA using the original BH_4 synthesis strain (*S. cerevisiae* GTPCH, *M. alpina* PTPS and SR) and the combinatorially optimized BH_4 synthesis strain (*E. coli* GTPCH, *M. alpina* PTPS and SR), both strains carrying tyrosine mono-oxygenase. Improving BH_4 production improves amino acid mono-oxidation. (**e**) Optimization of BH_4 biosynthesis. BH_4 synthesis pathway: *E. coli* GTPCH, *M. alpina* PTPS and SR. For the glucose system, all three enzymes were expressed from a single multicopy plasmid under control of constitutive promoters (P_{ADH1}, P_{TEF1}, and P_{HXT7}). (f) BH_4 biosynthetic pathway bottleneck identification. Biopterin production from galactose in yeast expressing each BH_4 biosynthetic enzyme from either a single-copy (s) or a multicopy (m) plasmid. All experiments were run in triplicate and shown are the means and standard deviations. GTPCH, GTP cyclohydrolase; PTPS, pyruvoyl tetrahydropterin synthase; SR, sepiapterin reductase.

dihydrobiopterin or biopterin before reaching the tyrosine mono-oxygenase. BH_4 must be synthesized intracellularly rather than exogenously fed to determine whether 0.74 mg/L BH_4 limits alkaloid biosynthesis.

So as not to limit alkaloid production due to insufficient BH_4, we combinatorially overexpressed four GTPCHs, four PTPSs, and three SRs in *S. cerevisiae* to identify a high-level BH_4-producing yeast strain. Among GTPCHs, we screened the enzymes from *E. coli*, *M. alpina*, *Homo sapiens*, and *S. cerevisiae*. *E. coli* GTPCH has a low K_M (0.02[50]−100[51] μM) and has been previously expressed in *S. cerevisiae*.[52] *H. sapiens* GTPCH has a pI of 5.6,[53] which could aid in its solubility, and it has also been expressed in *S. cerevisiae*.[52] Among PTPSs, we screened the enzymes from *M. alpina*, *Salmo salar*, the halophile *Salinibacter ruber*, and the bacteria *Phycisphaera mikurensis*. *S. salar* PTPS has a specific activity that is 50 times higher, and a K_M that is five times lower, than that of the canonical human PTPS.[54] We screened the putative PTPS from *S. ruber* because a structural homology model alignment with *S. salar* PTPS revealed that these enzymes have an almost identical active site, except that *S. ruber* PTPS has a catalytic aspartate rather than a cysteine residue[55] (SI Figure 3). We hypothesized that the aspartate's carboxylate functions as a better acid−base catalyst compared to cysteine's thiol group. We screened the predicted PTPS from *P. mikurensis* because its active site is almost identical to the well-studied *Rattus rattus* PTPS;[56] however, the *P. mikurensis* PTPS N-terminus has an additional ∼100 amino acids. A bioinformatics search revealed that *S. cerevisiae* lacked any PTPS homologue. Among SRs, we screened the SR from *M. alpina*, a predicted SR from the diatom *Thalassiosira*

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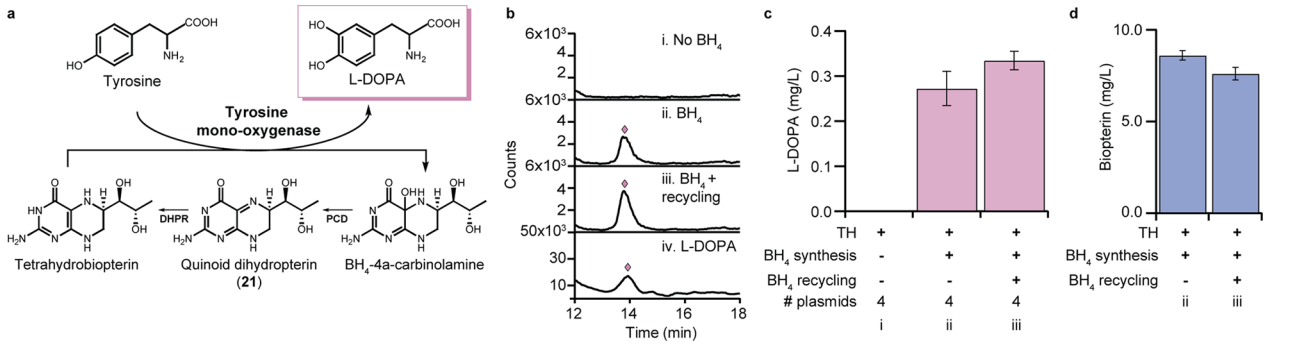

Figure 4. Microbial synthesis of L-DOPA *via* pterin-dependent tyrosine mono-oxidation. (a) Schematic representation of the BH_4 recycling pathway. (b) Representative LC traces for various L-DOPA production strains (extracted ion chromatograms for L-DOPA = m/z 198). Traces represent strains expressing (i) only tyrosine hydroxylase (TH), (ii) TH and the BH_4 synthesis pathway, and (iii) TH, the BH_4 synthesis pathway, and the BH_4 recycling pathway. Trace iv is commercial L-DOPA standard. Full windows of the spectra can be found in SI Figure 7a. (c) Production levels of L-DOPA from galactose in the presence (+) or absence (−) of the BH_4 synthesis pathway and/or BH_4 recycling pathway. (d) Production levels of biopterin from galactose in the presence (+) or absence (−) of the BH_4 recycling pathway. All experiments were run in triplicate and shown are the means and standard deviations. Strains carried four multicopy plasmids in which each gene was expressed from galactose inducible promoters (P_{GAL1} or P_{GAL10}). PCD, pterin-4a-carbinolamine dehydratase; DHPR, dihydropteridine reductase.

pseudonana, which, based on structural homology models is hypothesized to be NADH- rather than NADPH-dependent (SI Figure 4), and a putative SR from *S. cerevisiae* identified using bioinformatics.

The combination of *E. coli* GTPCH, *M. alpina* PTPS, and *M. alpina* SR resulted in the highest biopterin microbial synthesis, at 5.53 mg/L (Figure 3b, SI Figure 2). *E. coli* GTPCH outperformed all other GTPCHs, including the endogenous *S. cerevisiae* GTPCH. *H. sapiens* and *M. alpina* GTPCH resulted in the lowest biopterin production, with 3 of the 12 strains expressing *M. alpina* GTPCH producing undetectable levels of biopterin. PTPS expression showed graded levels of biopterin production with *M. alpina* ≈ *S. salar* > *S. ruber* > *P. mikurensis* in most cases. Expression of *M. alpina* SR resulted in statistically significant higher production of biopterin than the *S. cerevisiae* or *T. pseudonana* SRs in all 32 cases (all *P*-values < 0.015). No BH_4 or dihydrobiopterin were found in the medium or inside the cell, and negligible amounts of biopterin were found inside the cell, supporting diffusion of biopterin out of the cell (Figure 3c). As suspected, BH_4 levels had a limiting effect on pterin-dependent amino acid hydroxylation, judging by the performance of tyrosine mono-oxygenase in different BH_4-producing strains. L-DOPA production from the combinatorially optimized BH_4 synthesis strain (*E. coli* GTPCH, *M. alpina* PTPS and SR) resulted in 5-fold higher L-DOPA levels when compared to the initial BH_4 synthesis yeast strain (*S. cerevisiae* GTPCH (chromosomal), *M. alpina* PTPS and SR) (Figure 3d).

Optimization of Tetrahydrobiopterin Biosynthesis. We hypothesized that increasing the flux through the purine pathway should increase GTP levels and, in turn, BH_4 production. The parent yeast strain, W303, has a nonfunctional phosphoribosylaminoimidazole carboxylase (*ade2*) gene located upstream of GTP in the purine biosynthetic pathway (SI Figure 5). We generated a functional *Ade2* yeast strain and introduced the combinatorially optimized BH_4 synthesis pathway, achieving 7.81 mg/L of biopterin, a statistically significant improvement over the combinatorially optimized BH_4 synthesis pathway in the *ade2* strain (5.53 mg/L, *P* value < 0.04) (Figure 3e). To identify bottlenecks within the BH_4 pathway, we dosed the expression of each gene in the combinatorially optimized BH_4 synthesis pathway by expressing each gene from a single- or multi-copy plasmid using galactose-inducible promoters. The highest biopterin production was obtained when GTPCH was expressed from a multicopy plasmid with PTPS and SR expressed from single-copy plasmids, producing 9.13 mg/L of biopterin (Figure 3f). To help rationalize this result, we measured GTPCH, PTPS, and SR mRNA levels when expressed from single- or multi-copy plasmids (SI Figure 6). GTPCH mRNA levels were lowest when expressed from a multicopy plasmid, while PTPS and SR mRNA levels were lowest when expressed from a single-copy plasmid. Thus, the best BH_4-producing strain had the lowest mRNA levels for each of the three genes, hinting that overexpression of the three enzymes may be a burden to the system. Nevertheless, the increase in biopterin production was not statistically different from the 7.81 mg/L of biopterin produced when all enzymes were expressed from multicopy plasmids in the *Ade2* strain (Figure 3f). To produce BH_4 from glucose and to reduce plasmid burden,[57,58] the best enzyme combination was expressed in the *Ade2* strain from a single multicopy plasmid using constitutive promoters. Surprisingly, the single-plasmid BH_4 synthesis pathway resulted in only 7.69 mg/L of biopterin (Figure 3e), not statistically different from the biopterin production obtained from the three-plasmid galactose induced system (7.81 mg/L). It is possible that GTPCH, PTPS, and SR overexpression burden is more important than plasmid maintenance burden.

Tetrahydrobiopterin Recycling for Pterin-Dependent Amino Acid Mono-oxidation. Upon amino acid mono-oxidation, BH_4 is converted to BH_4-4a-carbinolamine (**20**). Previous work in *E. coli* has shown the BH_4 recycling pathway to be critical to ensure continuous supply of the BH_4 analogue MH_4 in pterin-dependent amino acid mono-oxidation.[31,35] To provide a continuous supply of BH_4 to the amino acid mono-oxygenases, we established a BH_4 recycling pathway in *S. cerevisiae*. In the recycling pathway, BH_4-4a-carbinolamine is converted back to BH_4 *via* the intermediate quinoid dihydrobiopterin (**21**) through consecutive reactions by pterin-4a-carbinolamine dehydratase (PCD) and dihydropteridine reductase (DHPR) (Figure 4a). We measured the effect of the *H. sapiens* BH_4 recycling pathway on the pterin-dependent mono-oxidation of tyrosine to L-DOPA, using the codon optimized *Mus musculus* tyrosine hydroxylase, in (1) the absence of the BH_4 biosynthetic and recycling pathways, (2)

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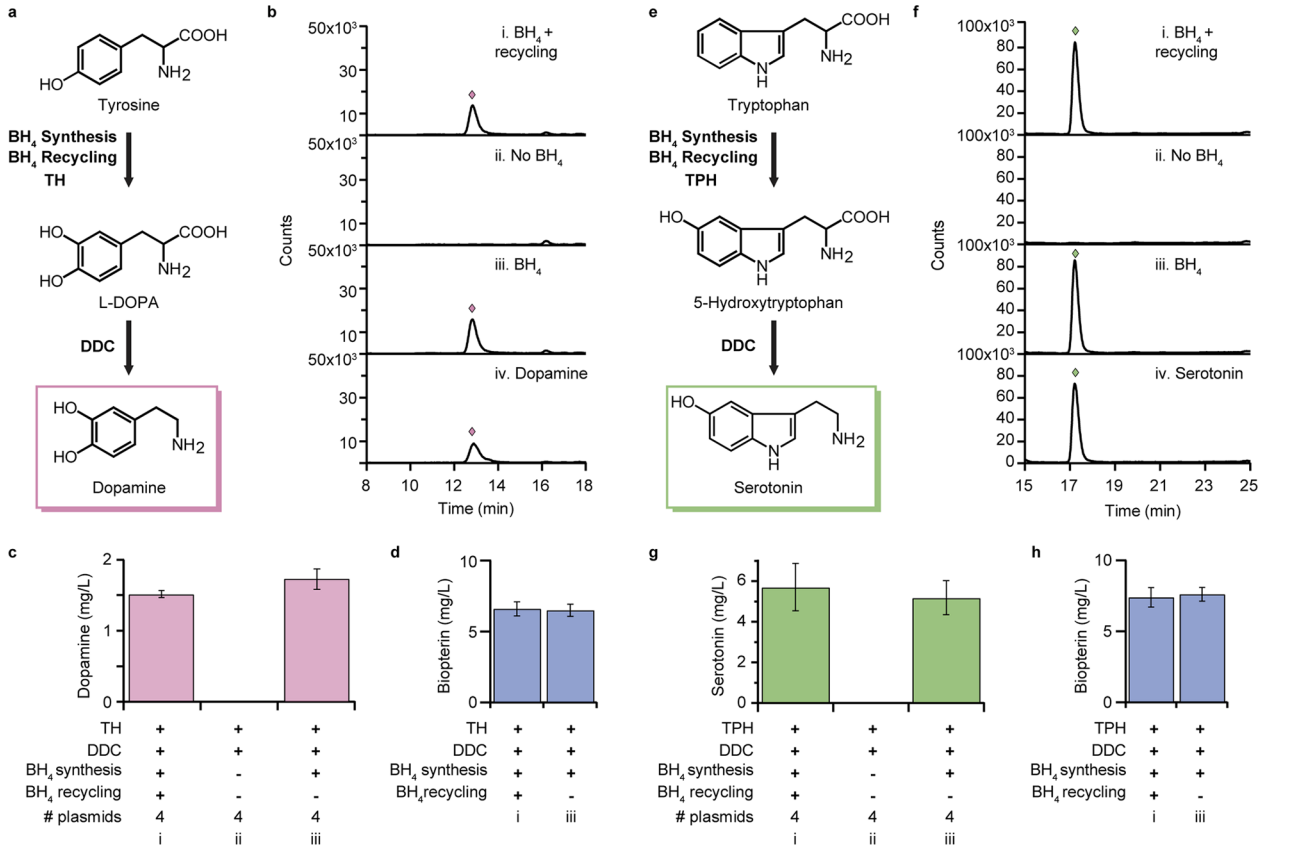

Figure 5. Microbial synthesis of biogenic amines *via* pterin-dependent mono-oxidation. (a) Schematic representation of dopamine biosynthesis. (b) Representative LC traces for various production strains (extracted ion chromatograms (EIC) for dopamine = m/z 154). Traces represent strains expressing (i) tyrosine hydroxylase (TH), aromatic-L-amino-acid decarboxylase (DDC), the BH_4 synthesis pathway, and the BH_4 recycling pathway, (ii) TH and DDC, and (iii) TH, DDC, and the BH_4 synthesis pathway. Trace iv is commercial dopamine standard. Full windows of the spectra can be found in SI Figure 7b. (c) Production levels of dopamine in the presence (+) or absence (−) of the BH_4 synthesis pathway, and/or the BH_4 recycling pathway. (d) Production levels of biopterin in the presence (+) or absence (−) of the BH_4 recycling pathway. (e) Schematic representation of serotonin biosynthesis. (f) Representative LC traces for various production strains (EIC for serotonin = m/z 177). Traces represent strains expressing (i) tryptophan hydroxylase (TPH), DDC, the BH_4 synthesis pathway, and the BH_4 recycling pathway, (ii) TPH and DDC, and (iii) TPH, DDC, and the BH_4 synthesis pathway. Trace iv is commercial serotonin standard. Full windows of the spectra can be found in SI Figure 7c. (g) Production levels of serotonin in the presence (+) or absence (−) of the BH_4 synthesis pathway and/or BH_4 recycling pathway. (h) Production levels of biopterin in the presence (+) or absence (−) of the BH_4 recycling pathway. All experiments were run in triplicate and shown are the means and standard deviations. Strains carried four multicopy plasmids in which each gene was expressed from galactose inducible promoters (P_{GAL1} or P_{GAL10}).

the presence of only the BH_4 biosynthetic pathway, and (3) the presence of both the BH_4 biosynthetic and recycling pathways (Figure 4b,c, SI Figure 7a). As expected, no L-DOPA was produced in the absence of the BH_4 biosynthetic and recycling pathways. In the presence of only the BH_4 biosynthetic pathway, L-DOPA was produced at 0.27 mg/L, while in the presence of both the BH_4 biosynthetic and recycling pathways, L-DOPA was produced at 0.33 mg/L. This increase in L-DOPA was not statistically significant, and the difference in biopterin levels in the presence or absence of the recycling pathway was not statistically significant either (Figure 4d). We conclude that high-level BH_4 synthesis eliminates the necessity of the BH_4 recycling pathway at the current rate of tyrosine mono-oxidation. To determine if tyrosine was limiting L-DOPA production, we supplemented the media with tyrosine and observed a slight increase in L-DOPA levels (SI Figure 8). Thus, improving tyrosine biosynthesis should increase L-DOPA.

Microbial Synthesis of Biogenic Amines *via* Pterin-Dependent Mono-oxidation. Biogenic amines are the immediate precursors to both MIAs and BIAs.[5] To microbially synthesize the BIA biogenic amine precursor dopamine from galactose, we engineered a yeast strain carrying the BH_4 biosynthetic and recycling pathways, tyrosine mono-oxygenase, and the codon optimized *Sus scrofa* aromatic L-amino-acid decarboxylase (DDC) (Figure 5a). This strain produced dopamine at 1.52 mg/L, while no dopamine was produced in the absence of the BH_4 biosynthetic and recycling pathways (Figure 5b,c, SI Figure 7b). To microbially synthesize the MIA biogenic amine serotonin from galactose, we engineered a yeast strain carrying the BH_4 biosynthetic and recycling pathways, a truncated codon optimized *H. sapiens* tryptophan hydroxylase,[59] and DDC (Figure 5e). This strain produced serotonin at 5.72 mg/L, while no serotonin was produced in the absence of the BH_4 synthesis and recycling pathways (Figure 5f,g, SI Figure 7c). In the presence of the amino acid mono-oxygenase, DDC, and the BH_4 biosynthetic pathway, but in the absence of the BH_4 recycling pathway, dopamine was produced at 1.73 mg/L, while serotonin was produced at 5.18 mg/L (Figure 5c,g). As with the production of L-DOPA, the BH_4 recycling

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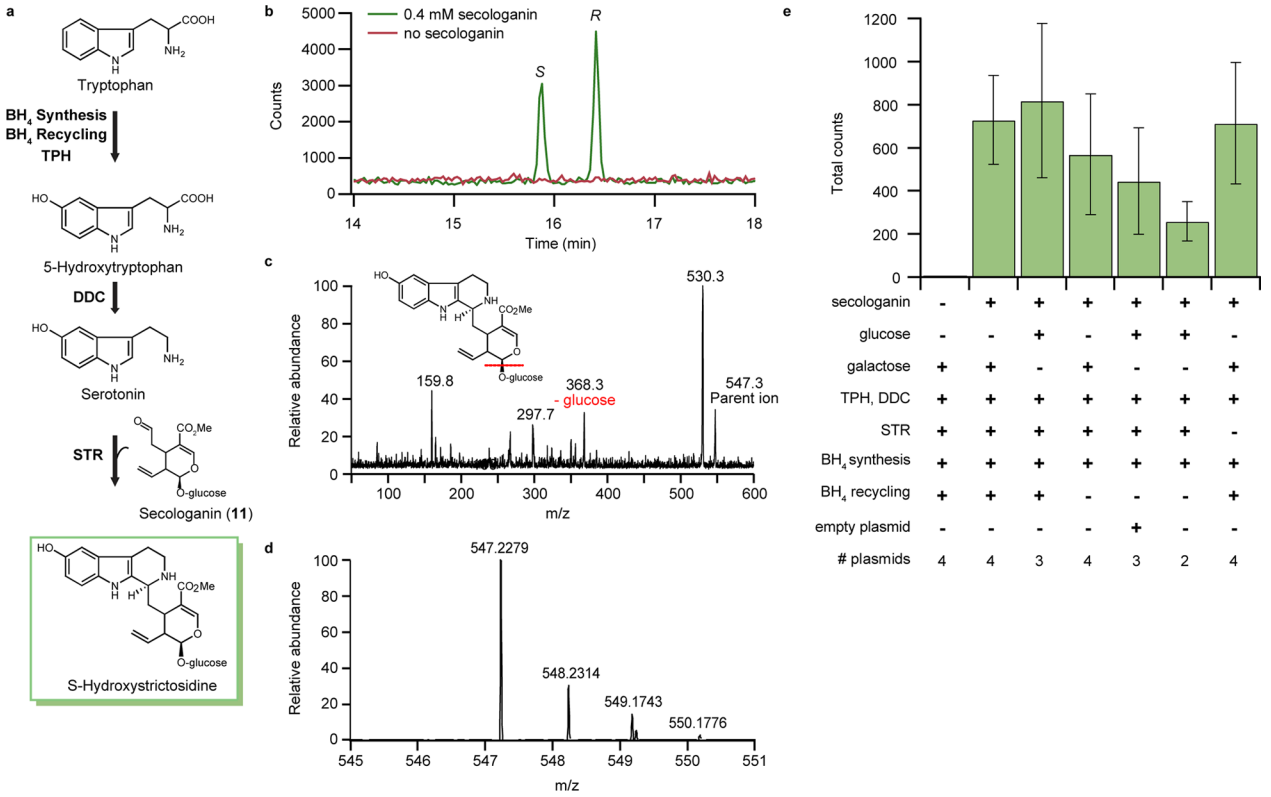

Figure 6. Microbial synthesis of the modified MIA 10-hydroxystrictosidine. (a) Schematic of hydroxystrictosidine biosynthesis. (b) Representative LC trace (multiple reaction monitoring hydroxystrictosidine 547.60 → 530.00 transition) for the yeast strain (PPY650) carrying tryptophan hydroxylase, aromatic-L-amino-acid decarboxylase, strictosidine synthase, and the BH_4 biosynthesis and recycling pathways in the presence or absence of 0.4 mM secologanin. S = (*S*)-hydroxystrictosidine; R = (*R*)-hydroxystrictosidine. Full window of the spectra can be found in SI Figure 10. (c) Tandem mass spectrum of microbially synthesized *S*-hydroxystrictosidine. (d) High-resolution mass spectrum of microbially produced S-hydroxystrictosidine. (e) (*S*) and (*R*)-hydroxystrictosidine production in *S. cerevisiae*. DDC, aromatic-L-amino-acid decarboxylase; TPH, tryptophan hydroxylase; STR, strictosidine synthase.

pathway had no statistically significant effect on biogenic amine production. The dopamine- and serotonin-producing strains had similar levels of biopterin with and without the BH_4 recycling pathway (Figure 5d,h). To determine if tryptophan was limiting serotonin production, we supplemented the media with tryptophan. Indeed, increasing extracellular tryptophan levels increased serotonin production (SI Figure 9). Thus, improving yeast tryptophan biosynthesis should increase serotonin levels.

Microbial Synthesis of the Modified MIA 10-Hydroxystrictosidine. Although several microbial strains have been engineered for the production of BIAs,[19−25] engineering of MIA microbial platforms has lagged behind.[60] Further, to our knowledge no modified alkaloid has been produced microbially to date. Microbial synthesis of modified alkaloids has the potential to generate more amenable intermediates for chemical derivatization to obtain final therapeutics. Therefore, we focused our pterin-dependent biogenic amine-producing strain toward the production of the modified MIA hydroxystrictosidine. To microbially synthesize hydroxystrictosidine from galactose and secologanin, we used the serotonin-producing strain, carrying the BH_4 recycling pathway and expressing the *Ophiorrhiza pumila* strictosidine synthase[61] with the vacuolar tag removed so as to avoid enzyme secretion[62] (Figure 6a). The strain produced both *R*- and *S*-hydroxystrictosidine isomers. (Figure 6b, SI Figure 10). Given that hydroxystrictosidine is not commercially available, we characterized the compounds using tandem mass spectrometry and high resolution mass spectrometry (Figure 6c,d, SI Figure 11). To improve upon the inducible four-plasmid system, which has drawbacks in terms of carbon source, plasmid burden, stability, and the reuse of promoters, we engineered a three-plasmid system using multicopy plasmids with the enzymes under control of constitutive promoters. The four- and three-plasmid systems produced similar levels of hydroxystrictosidine (Figure 6e, Table 1). Interested in whether the BH_4 recycling pathway had an effect on hydroxystrictosidine levels, we removed the recycling pathway in both the four- and three-plasmid systems and saw that the BH_4 recycling pathway had no significant impact on hydroxystrictosidine production. Given this observation, we engineered a two-plasmid system without the BH_4 recycling pathway; however, this system also failed to improve hydroxystrictosidine production. Interestingly, all the hydroxystrictosidine-producing yeast strains resulted in a mixture of *R*- and *S*-hydroxystrictosidine isomers even though previous *in vitro* work feeding tryptamine and secologanin to strictosidine synthase at pH = 7 resulted in the stereoselective synthesis of *S*-strictosidine.[61] Removing strictosidine synthase from the hydroxystrictosidine-producing strain resulted in similar *R*- and *S*-hydroxystrictosidine levels (SI Figure 12). This indicated that, at the low pH of the yeast medium (pH = 5−3), secologanin and serotonin were coupling chemically rather than enzymatically to produce a mixture of hydroxystrictosidine isomers.[63] Indeed, strictosidine synthase retains less than one tenth of its activity at pH = 4−3[64] and has 3 orders of

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Table 1. Overview of the Microbial Synthesis of L-DOPA, Dopamine, Serotonin and 10-Hydroxystrictosidine[a]

Strain	BH_4 Synthesis Pathway	BH_4 Recycling Pathway	Alkaloid Synthesis Patway	# Plasmids	Substrate(s)	Product	Production
PPY946			TH	4	Galactose	L-DOPA	nd
PPY646	GTPCH PTPS SR	PCD DHPR	TH	4	Galactose	L-DOPA	0.33 ± 0.02 mg/L
PPY679	GTPCH PTPS SR		TH	4	Galactose	L-DOPA	0.27 ± 0.04 mg/L
PPY947			TH DDC	4	Galactose	Dopamine	nd
PPY658	GTPCH PTPS SR	PCD DHPR	TH DDC	4	Galactose	Dopamine	1.52 ± 0.05 mg/L
PPY743	GTPCH PTPS SR		TH DDC	4	Galactose	Dopamine	1.73 ± 0.14 mg/L
PPY948			TPH DDC	4	Galactose	Serotonin	nd
PPY649	GTPCH PTPS SR	PCD DHPR	TPH DDC	4	Galactose	Serotonin	5.72 ± 1.18 mg/L
PPY741	GTPCH PTPS SR		TPH DDC	4	Galactose	Serotonin	5.18 ± 0.84 mg/L
PPY650	GTPCH PTPS SR	PCD DHPR	TPH DDC STR	4	Galactose Secologanin	Hydroxystrictosidine	728.8 ± 206.2 counts
PPY955	GTPCH PTPS SR		TPH DDC STR	4	Galactose Secologanin	Hydroxystrictosidine	570.6 ± 280.6 counts
PPY744	GTPCH PTPS SR	PCD DHPR	TPH DDC STR	3	Glucose Secologanin	Hydroxystrictosidine	819.4 ± 357.3 counts
PPY748	GTPCH PTPS SR		TPH DDC STR	3	Glucose Secologanin	Hydroxystrictosidine	444.7 ± 246.5 counts
PPY740	GTPCH PTPS SR		TPH DDC STR	2	Glucose Secologanin	Hydroxystrictosidine	258.7 ± 91.3 counts
PPY649	GTPCH PTPS SR	PCD DHPR	TPH DDC	4	Galactose Secologanin	Hydroxystrictosidine	713.9 ± 281.9 counts

[a]Arrows represent the presence of the enzyme; nd = not detectable. Amount produced is represented by the mean ± standard deviation for samples run in triplicate. GTPCH, GTP cyclohydrolase; PTPS, pyruvoyl tetrahydropterin synthase; SR, sepiapterin reductase; PCD, pterin-4a-carbinolamine dehydratase; DHPR, dihydropteridine reductase; TPH, tryptophan hydroxylase; TH, tyrosine hydroxylase; DDC, aromatic-L-amino-acid decarboxylase; STR, strictosidine synthase.

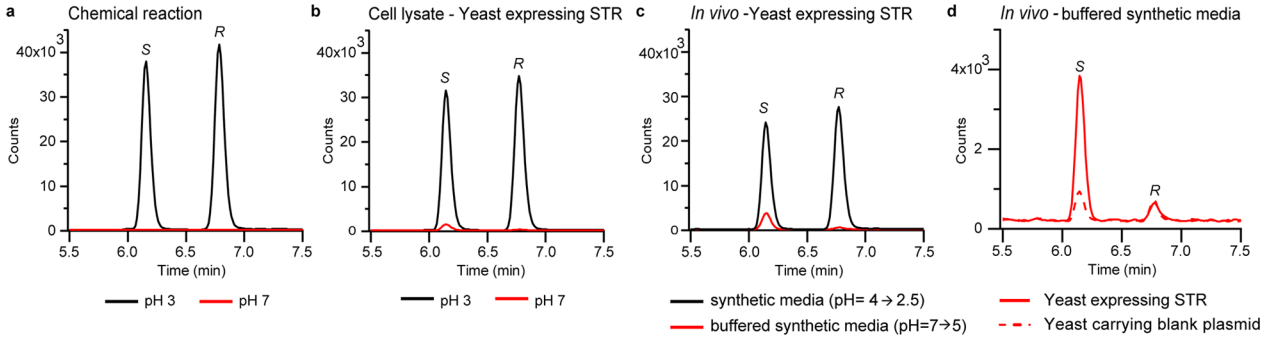

Figure 7. Analysis of the 10-hydroxystrictosidine isomer ratio. All reactions contain 0.4 mM each of secologanin and serotonin. LC traces (extracted ion chromatograms for hydroxystrictosidine = m/z 547) for (a) the chemical reaction in phosphate buffer at pH = 3 (black) or pH = 7 (red); (b) the reaction in cell lysate of yeast expressing strictosidine synthase (PPY827) adjusted to pH = 3 (black) or pH = 7 (red); (c) *in vivo* reaction using intact yeast cells expressing strictosidine synthase (PPY827) in standard yeast media (black) or pH = 7 buffered media (red); and (d) *in vivo* reaction using intact yeast cells expressing either strictosidine synthase (PPY827, solid red line) or yeast expressing a blank plasmid (PPY828, dotted red line) in pH = 7 buffered media. Full windows of the spectra can be found in SI Figure 13. Multiple reaction monitoring of Figure 7d can be found in SI Figure 15. STR, strictosidine synthase.

magnitude less catalytic activity with serotonin when compared to tryptamine.[65]

Determining Strictosidine Synthase Functionality. To determine if strictosidine synthase was functionally expressed in the hydroxystrictosidine-producing yeast strain, we examined the level of spontaneous and enzymatic hydroxystrictosidine synthesis under different conditions. First, we confirmed that spontaneous condensation of serotonin and secologanin does not occur at pH = 7, but does at pH = 3, producing both hydroxystrictosidine isomers (Figure 7a, SI Figure 13a), explaining why *in vitro* strictosidine synthase studies at pH = 7 do not exhibit spontaneous condensation and produce only the S-isomer. Next, we tested hydroxystrictosidine formation using the lysate of yeast cells expressing strictosidine synthase. When the lysate was placed at pH = 3 and fed serotonin and secologanin, both hydroxystrictosidine isomers were formed, while the same experiment at pH = 7 resulted in only the S-isomer (Figure 7b, SI Figure 13b), demonstrating that strictosidine synthase is functionally expressed in yeast. Isomer identification was determined due to the fact that strictosidine synthase is known to form only the S-isomer[61] while the spontaneous chemical condensation produces both R- and S-isomers, with the R-isomer being the major product.[63] Next, we used intact yeast cells expressing strictosidine synthase, fed serotonin and secologanin, and cultured the cells for 136 h using standard (pH = 4−2.5, SI Figure 14) or buffered (pH = 7−5, SI Figure 14) synthetic complete media. Yeast cells in the buffered media resulted in the synthesis of only *S*-

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hydroxystrictosidine (Figure 7c, SI Figure 13c). Finally, we showed that strictosidine synthase is necessary for *S*-hydroxystrictosidine production in yeast and that the reaction was not catalyzed by an endogenous yeast enzyme (Figure 7d, SI Figure 13d, SI Figure 15). These results demonstrate that strictosidine synthase is functionally expressed in the hydroxystrictosidine-producing yeast strain, that the enzymatic reaction leading to the S-isomer is taking place intracellularly, and that secologanin is crossing the yeast cell membrane. In standard yeast synthetic complete media, however, the spontaneous coupling of serotonin and secologanin is the predominant hydroxystrictosidine forming reaction.

DISCUSSION

Together, these results establish the first microbial synthesis of a modified alkaloid, specifically a modified MIA, representing an alkaloid family with important medicinal compounds, including anticancer, antimalarial, and antiarrhythmic agents. We produced hydroxystrictosidine from glucose and secologanin in *S. cerevisiae* by leveraging the pterin-dependent mono-oxidation of tryptophan using microbially produced BH_4 as a cofactor. Pterin-dependent mono-oxidation of tryptophan was necessary as no known natural enzyme oxidizes tryptophan akin to how tyrosinase oxidizes tyrosine, and plant enzymes used to convert tryptophan to serotonin can be promiscuous. We chose *S. cerevisiae* as the microbial host due to its robustness to industrial conditions, which makes this MIA-producing platform attractive for continued use. Further, the presence of compartments in *S. cerevisiae* with unique pH values and metal concentrations can be exploited to express downstream MIA pathway enzymes at the ideal conditions for maximal enzymatic activity or to prevent byproduct formation. Finally, although in this work we fed secologanin to the microbe, in the future, secologanin can also be biosynthesized from glucose as the nine-step secologanin biosynthetic pathway from geranyldiphosphate is known and has been reconstituted in yeast.[60,66]

We were surprised by the non-necessity of the BH_4 recycling pathway for amino acid mono-oxidation and ultimately hydroxystrictosidine production, even though a BH_4 recycling pathway was shown to be necessary for the production of hydroxytyrosol[35] and hydroxytryptophan[31] in *E. coli* when using the cofactor analogue MH_4. In our engineered yeast strain, BH_4 synthesis alone is sufficient to drive robust amino acid mono-oxidation, and aromatic amino acid levels are currently limiting alkaloid production. Using the pterin-dependent mono-oxidation strategy, L-DOPA is produced to 0.33 mg/L, which is comparable to L-DOPA production in yeast using a tyrosinase at ~0.1 mg/L[29]. Interestingly, L-DOPA production was higher (~1 mg/L) in the BH_4-producing strain synthesizing slightly less BH_4 (5.53 mg/L *vs* 7.81 mg/L) due to a nonfunctional mutation in the *ade2* gene. The resource allocation for the increased BH_4 production may be detrimental to the production of tyrosine, and, thus limiting for L-DOPA. Therefore, matching the levels of the BH_4 cofactor supply to the demand of the amino acid mono-oxygenase should enable further improvement in the yield of amino acid mono-oxidation and, in turn, in BIA or MIA production. Increasing the tyrosine supply and dynamically regulating the BH_4 levels to match the demand of the amino acid mono-oxygenase may enable the production of L-DOPA at the levels achieved in *E. coli*, 293 mg/L[21].

Using standard yeast media, both *R*- and *S*-hydroxystrictosidine isomers are produced; through testing various reaction conditions, we conclude that strictosidine synthase is functional in our strain, although at the low pH of the yeast media the chemical condensation of serotonin and secologanin prevails. While the enzymes in the MIA pathway are likely to prefer one isomer over the other for further processing, synthesis of both hydroxystrictosidine isomers may not be detrimental to the production of more complex MIAs as later enzymes in MIA pathways, such as strictosidine glucosidase, are able to turn over the *R*-isomer, albeit at lower rates when compared to the *S*-isomer.[67,68] Further, enzymes capable of turning over alkaloid isomer mixtures have also been seen in other alkaloid families, such as BIAs.[19] Alternatively, later enzymes in the MIA pathway may be able to convert only one isomer, as in the case of the berberine bridge enzyme in the BIA pathway,[69] thus reducing the concerns of producing a mixture of hydroxystrictosine isomers early on in the pathway.

There are several opportunities and some key challenges to improving the yield of amino acid mono-oxidation and, in turn, MIA production. First, we show that tyrosine and tryptophan levels limit the system; thus, increasing aromatic amino acid supply *via* introduction of feedback-resistant enzyme mutants in aromatic amino acid metabolism, such as Aro4,[70] or utilizing a tyrosine overproducing strain,[71] should enable straightforward increases in amino acid mono-oxidation. Second, our system does not currently take advantage of the power of the BH_4 recycling system, as biogenic amine levels are similar in the presence and absence of the recycling system. Screening or engineering aromatic amino acid mono-oxidases with increased *in vivo* activity should also increase aromatic amino acid mono-oxidation. Finally, the BH_4 synthesis and recycling pathways could be integrated to reduce plasmid burden, and the MIA pathway enzymes could be colocalized to increase flux through the pathway.[72] The major challenge with any of these potential approaches to improving the yield of aromatic amino acid mono-oxidation is the low-throughput of the chromatography-based screens required to detect and quantify these compounds, which take close to one hour per sample. Biosensors for key compounds in the pathway, such as hydroxylated amino acids,[29] biogenic amines, or, ideally, the terminal MIAs, should enable the faster optimization of this and other metabolic pathways with products that are neither colorimetric nor fluorescent. A general obstacle is that a new biosensor must be engineered for every chemical of interest, and current strategies to rapidly engineer user-specified chemical biosensors are at the proof-of-principal stage[73] or still on the drawing board.

The strains reported in this work could be further optimized for production of (1) BH_4, itself being a treatment for phenylketonuria,[74] (2) hydroxytryptophan, serotonin, and serotonin derivatives, key compounds in depression treatment and studies,[75−77] (3) DOPA and dopamine, important compounds related to Parkinson's disease,[78] and (4) advanced alkaloids, including anticancer agents. The BH_4-producing yeast strain could be used for the synthesis of both MIAs and BIAs, by introducing different amino acid mono-oxygenases. For example, the dopamine-producing strain can be rapidly adapted to produce BIAs by using norcoclaurine synthase and hydroxyphenylacetaldehyde to produce norcoclaurine (**22**). Ultimately, we envision the MIA-producing strain being used in the biosynthesis of modified and non-natural MIAs or as a plant pathway discovery tool to identify unknown steps in MIA biosynthetic pathways. For instance, we can use the hydroxystrictosidine platform to screen cDNA libraries of

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plant tissues known to overproduce MIAs to rapidly identify missing steps in MIA biosynthetic pathways to medically important alkaloids.

METHODS

Microbial Synthesis of Tetrahydrobiopterin. Overnight cultures of strains PPY750, 752−793, and 797−810 in synthetic complete media with 2% glucose lacking histidine, tryptophan, and leucine (SD (HWL^-)) were used to inoculate 5 mL of synthetic complete media with 2% galactose lacking histidine, tryptophan, and leucine (SCgal (HWL^-)) to OD_{600} = 0.1 and incubated for 136 h at 30°C (250 rpm). Overnight culture of strain PPY749 in synthetic complete media with 2% glucose lacking histidine (SD (H^-)) was used to inoculate 5 mL of synthetic complete media with 2% galactose lacking histidine (SCgal (H^-)) to OD_{600} = 0.1 and incubated for 136 h at 30°C (250 rpm). Overnight culture of strain PPY751 in synthetic complete media with 2% glucose lacking histidine and tryptophan (SD (HW^-)) was used to inoculate 5 mL of synthetic complete media with 2% galactose lacking histidine and tryptophan (SCgal (HW^-)) to OD_{600} = 0.1 and incubated for 136 h at 30°C (250 rpm). After incubation, cultures were centrifuged for 5 min at 3230*g*, the supernatant was filtered, vanillin was added as an internal standard and samples were analyzed *via* LC−MS. For quantification of biopterin in L-DOPA, dopamine, and serotonin-producing strains, 5-chlorotryptamine was used as an internal standard.

Microbial Synthesis of L-DOPA, Dopamine, Serotonin, and Hydroxystrictosidine. Overnight cultures of strains PPY646, 649−650, 658, 679, 741, 743, 946−948, and 955 in synthetic complete media with 2% glucose lacking histidine, tryptophan, leucine, and uracil (SD ($HWLU^-$)) were used to inoculate 5 mL of synthetic complete media with 2% galactose lacking histidine, tryptophan, leucine, and uracil (SCgal ($HWLU^-$)) to OD_{600} = 0.1. Overnight cultures of strains PPY744 and 748 in SD (HWU^-) were used to inoculate 5 mL of fresh SD (HWU^-) to OD_{600} = 0.1. Overnight culture of strain PPY740 in SD (HW^-) was used to inoculate 5 mL of fresh SD (HW^-) to OD_{600} = 0.1. For hydroxystrictosidine production (strains PPY649, 650, 740, 744, 748, and 955), secologanin (solution in water) was added at the time of inoculation to a final concentration of 0.4 mM (150 mg/L). After inoculation, all strains were incubated for 136 h at 30°C (250 rpm). The cultures were then centrifuged for 5 min at 3230*g*, the supernatant was filtered, 5-chlorotryptamine (L-DOPA, dopamine, serotonin) or vanillin (hydroxystrictosidine) was added as an internal standard, and the samples were analyzed *via* LC−MS.

Biopterin Quantification. LC−MS analysis was completed using an Agilent 1100/1260 series system equipped with a 1260 ALS autosampler and a 6120 Single Quadrupole LC−MS with a Poroshell 120 SB-Aq 3.0 mm × 100 mm × 2.7 μm column and an electrospray ion source. LC conditions: solvent A—150 mM acetic acid with 0.1% formic acid; solvent B—methanol with 0.1% formic acid. Gradient: 4 min ramp from 95%:5%:0.2 (A:B:flow rate in mL/min) to 70%:30%:0.2, 6 min ramp to 40%:60%:0.2, 2 min ramp to 2%:98%:0.2, 2 min ramp to 2%:98%:0.5, 4 min at 2%:98%:0.5, 1 min ramp to 95%:5%:0.5, 7 min at 95%:5%:0.5, and 1.5 min post time. MS acquisition (positive ion mode) included 25% scan from *m*/*z* 100−600, 25% scan from *m*/*z* 230−260, 25% scan from *m*/*z* 145−165, and 25% selected ion monitoring (SIM) for BH_4 (*m*/*z* 242.1), dihydrobiopterin (*m*/*z* 240.1), biopterin (*m*/*z* 238.1), and vanillin (*m*/*z* 153.1). Quantitation was performed by obtaining the area under the peak in the extracted ion chromatogram (EIC) for the desired *m*/*z* value from the SIM signal. For biopterin quantification in L-DOPA-, dopamine-, and serotonin-producing strains, 5-chlorotryptamine (*m*/*z* 195.1) was used as an internal standard instead of vanillin. Area was converted to concentration using standard curves produced from commercially available biopterin. Retention times were determined using commercially available standards.

Quantification of L-DOPA, Dopamine, and Serotonin. LC−MS system and solvent composition was the same as the one used in the analysis of biopterin. LC gradient: 8 min ramp from 95%:5%:0.05 to 70%:30%:0.05, 6 min ramp to 40%:60%:0.05, 1 min ramp to 40%:60%:0.1, 9 min ramp to 2%:98%:0.1, 1 min at 2%:98%:0.1, 5 min ramp to 2%:98%:0.3, 0.1 min ramp to 2%:98%:0.5, 3.9 min at 2%:98%:0.5, 1 min ramp to 95%:5%:0.5, 7 min at 95%:5%:0.5, and 3.5 min post time. MS acquisition (positive ion mode) included 33% scan from *m*/*z* 100−600, 33% scan from *m*/*z* 120−240, and 33% SIM for L-DOPA (*m*/*z* 198.2), dopamine (*m*/*z* 154.2), hydroxytryptophan (*m*/*z* 221.2), serotonin (*m*/*z* 177.2), and 5-chlorotryptamine (*m*/*z* 195.1). Quantitation was performed by obtaining the area under the peak in the EIC for the desired *m*/*z* value from the SIM signal. Area was converted to concentration using standard curves produced from commercially available L-DOPA, dopamine and serotonin dissolved in media taken from a culture of strain PPY810 grown under the same conditions as production samples. Traces used for the L-DOPA standard curve were background subtracted using just media. Retention times were determined using commercially available standards.

Analysis of Hydroxystrictosidine. High resolution mass spectrometry (HRMS) and tandem mass spectrometry (MS−MS) analysis of hydroxystrictosidine was performed at the Mass Spectrometry Facility at Georgia Tech. MS−MS was done using a Waters Quattro LC mass spectrometer with a Gemini 2 mm × 150 mm × 5 μm C18 column from Phenomenex. LC conditions: solvent A—95%:5% water:acetonitrile; solvent B—5%:95% water:acetonitrile. Gradient: 7 min at 100%:0% (A:B), 37 min ramp to 0%:100%, 8 min at 0%:100%, 1 min ramp to 100%:0%, and 7 min at 100%:0%. Flow rate was 0.2 mL/min. HRMS was done using a Thermo LTQ Orbitrap XL equipped with a Nano ACQUITY UPLC with a BEH130 300 μm × 100 mm × 1.7 μm C8 column from Waters. Solvent A—10 mM ammonium acetate in water; solvent B—acetonitrile. Gradient: 5 min at 95%:5% (A:B), 40 min ramp to 70%:30%, 5 min at 70%:30%, 2 min ramp to 5%:95%, 3 min at 5%:95%, 1 min ramp to 95%:5%, and 4 min at 95%:5%. Flow rate was 8 μL/min. Multiple Reaction Monitoring (MRM) was done on the Waters Quattro LC mass spectrometer using the same column and LC gradient using solvent A—95%:5%:0.1% water:acetonitrile:formic acid; solvent B—5%:95%:0.1% water:acetonitrile:formic acid. MRM parameters: hydroxystrictosidine: transition 547.60 → 530.00, cone voltage 20 V, collision energy 35 eV; transition 547.60 → 298.00, cone voltage 20 V, collision energy 35 eV. Vanillin: transition 152.80 → 92.80, cone voltage 25 V, collision energy 15 eV; transition 152.80 → 124.80, cone voltage 25 V, collision energy 15 eV. Camptothecin: transition 349.10 → 305.00, cone voltage 45 V, collision energy 35 eV; transition 349.10 → 220.00, cone voltage 45 V, collision energy 40 eV. Reported hydroxystrictosidine counts were obtained using 547.60 → 530.00 transition. Vanillin internal standard transition used 152.80 → 92.80.

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Hydroxystrictosidine Isomer Ratios. For the chemical reactions, secologanin and serotonin were mixed to a final concentration of 0.4 mM each in pH = 3 or pH = 7 phosphate buffer (135 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4). Solutions were mixed and incubated for 136 h at 30°C (250 rpm). After incubation, solutions were analyzed using LC−MS. For lysate experiments, an overnight culture of PPY827 in synthetic complete media with 2% glucose lacking uracil (SD(U^-)) was used to inoculate 5 mL of synthetic complete media with 2% galactose lacking uracil SCgal (U^-) to OD_{600} = 0.1. The culture was incubated for 24 h at 30 °C (250 rpm). After incubation, the culture was centrifuged at 3230*g* for 5 min, the supernatant was removed, and the pellet was resuspended in 1.5 mL phosphate buffer. The pellet was lysed by sonication using a Misonix Sonicator 3000 at 5.0 output level for 20 s, 20 s rest, for a total six pulses. The lysate was centrifuged, and supernatant was collected. The pH of the lysate was adjusted to either pH = 3 or pH = 7, and secologanin and serotonin were added to a final concentration of 0.4 mM each. After mixing, the lysates were incubated for 136 h at 30°C (250 rpm), after which the lysates were analyzed using LC−MS. For *in vivo* intact cell experiments, overnight cultures of strains PPY827 and PPY828 in SD (U^-) were used to inoculate 5 mL of SCgal (U^-) or SCgal (U^-) buffered with 25 mM K_2HPO_4 (pH = 7) to OD_{600} = 0.1. Secologanin and serotonin were added to a final concentration of 0.4 mM each and the cultures were incubated for 136 h at 30 °C (250 rpm). After incubation, all cultures were centrifuged for 5 min at 3230*g*, and the supernantant was filtered and analyzed *via* LC−MS. The column compartment was kept constant at 28°C. LC−MS analysis was completed on the Agilent system described above. Gradient: 0.25 min ramp from 95%:5% (A:B) to 70%:30%, 4.75 min ramp to 68%:32%, 2 min ramp to 30%:70%, 1 min at 30%:70%, 0.50 min ramp to 95%:5%, and 5.5 min at 95%:5%. Flow rate was 0.4 mL/min. MS acquisition (positive ion mode) included 30% scan from *m*/*z* 100−600 and 70% SIM for ions related to alkaloid formation (dopamine, *m*/*z* 154; tryptamine, *m*/*z* 161; serotonin, *m*/*z* 177; tyrosine, *m*/*z* 182; L-DOPA, *m*/*z* 198; tryptophan, *m*/*z* 205; 5-hydroxytryptophan, *m*/*z* 221; strictosidine, *m*/*z* 531; hydroxystrictosidine, *m*/*z* 547).

■ ASSOCIATED CONTENT

Supporting Information

Materials and Methods. Table of strains (SI Table 1); plasmids (SI Table 2); primers (SI Table 3). Stereochemistry of pterin cofactors (SI Figure 1); combinatorial production of biopterin (SI Figure 2); structural alignment of *S. ruber* and *S. salar* PTPS (SI Figure 3); structural alignment of *M. alpina* and *T. pseudonana* SR (SI Figure 4); purine biosynthetic pathway (SI Figure 5); GTPCH, PTPS, and SR mRNA levels (SI Figure 6); full windows of LC traces in Figures 4b, 5b, 5f (SI Figure 7); effect of tyrosine on L-DOPA production (SI Figure 8); effect of tryptophan on serotonin production (SI Figure 9); full window of multiple reaction monitoring for Figure 6b (SI Figure 10); mass spectrometry characterization of hydroxystrictosidine isomers (SI Figure 11); isomer ratios produced in the presence or absence of strictosidine synthase (SI Figure 12); full windows of LC traces in Figure 7 (SI Figure 13); pH of media over time (SI Figure 14); full window for multiple reaction monitoring analysis for Figure 7d (SI Figure 15); gene sequences used in this study. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.5b00025.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

MIA, monoterpene indole alkaloid; BIA, benzylisoquinoline alkaloid; BH_4, tetrahydrobiopterin; GTPCH, GTP cyclohydrolase I; PTPS, pyruvoyl tetrahydropterin synthase; SR, sepiapterin reductase; PCD, pterin-4-alpha-carbinolamine dehydratase; DHPR, dihydropteridine reductase; L-DOPA, L-3,4-dihydroxyphenylalanine; TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase; DDC, aromatic-L-amino-acid decarboxylase; STR, strictosidine synthase

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