Engineering a microbial biosynthesis platform for de novo production of tropane alkaloids

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Tropane alkaloids (TAs) are a class of phytochemicals produced by plants of the nightshade family used for treating diverse neurological disorders. Here, we demonstrate de novo production of tropine, a key intermediate in the biosynthetic pathway of medicinal TAs such as scopolamine, from simple carbon and nitrogen sources in yeast (Saccharomyces cerevisiae). Our engineered strain incorporates 15 additional genes, including 11 derived from diverse plants and bacteria, and 7 disruptions to yeast regulatory or biosynthetic proteins to produce tropine at titers of 6 mg/L. We also demonstrate the utility of our engineered yeast platform for the discovery of TA derivatives by combining biosynthetic modules from distant plant lineages to achieve de novo production of cinnamoyltropine, a non-canonical TA. Our engineered strain constitutes a starting point for future optimization efforts towards realizing industrial fermentation of medicinal TAs and a platform for the synthesis of TA derivatives with enhanced bioactivities.
Tropine alkaloids (TAs) are anticholinergic secondary metabolites produced by Atropa, Duboisia, and other genera of the nightshade family (Solanaceae). Several TAs, including atropine, hyoscyamine, and scopamine, are classified as essential medicines by the World Health Organization for treatment of organophosphate and nerve agent poisoning, gastrointestinal spasms, cardiac arrhythmia, and symptoms of Parkinson’s disease. TAs are sourced primarily via cultivation in Australia of Duboisia species, in which they accumulate to 0.2–4% by weight in plant tissue. Geographical restriction and reliance on monocultures renders the TA supply vulnerable to pests, changes in land use, and climate. No total chemical syntheses for TAs are sufficiently economical for industrial use due to challenging stereochemistries. Efforts to improve TA production in native species and engineer production via overexpression of rate-limiting enzymes in transgenic hairy root cultures have had limited success. Limited tools for genetic manipulation of Solanaceae, long generation times, and challenges with adapting hairy root cultures to large-scale bioreactors makes engineering plant hosts cost- and labor-intensive.

Baker’s yeast (Saccharomyces cerevisiae) is an attractive platform for industrial production of plant natural products with smaller resource and time requirements than plant-based supply chains. In addition to genetic tractability, metabolic plasticity, and compatibility with large-scale cultivation in bioreactors, yeast provides many requisite endomembrane structures and subcellular compartments for reconstitution of plant pathways with multiple cytochrome P450 enzymes. Yeast-based cellular factories have been described for terpenoids such as artemisinic acid, phenylpropanoids such as resveratrol and naringenin, benzylisoquinoline alkaloids such as hydrocodone and noscapine, and monoterpene indole alkaloids such as strigiosidine.

One challenge preventing a microbial production platform for TAs has been the lack of a fully characterized biosynthetic pathway. Medicinally relevant TAs, such as hyoscyamine and scopamine, consist of an arginine-derived 8-azabicyclo[3.2.1]octane (tropine) moiety esterified with a phenylalanine-derived phenylactate group by an unknown acyltransferase mechanism. The product, littorine, undergoes an internal free radical-mediated rearrangement catalyzed by a cytochrome P450 (CYP80F1) to produce hyoscyamine aldehyde, which is reduced to hyoscyamine by an unidentified dehydrogenase or reductase. Finally, a bifunctional 2-oxoglutarate/Fe(II)-dependent hyoscyamine 6β-hydroxylase/oxygenase (H6H) catalyzes the conversion of hyoscyamine to scopamine. Although the biosynthetic steps from arginine to the intermediate N-methylpyrrolinium (NMPy) were established over decades of work, the enzymes responsible for converting NMPy to tropine remained elusive. Recently, researchers reported the discovery of an unusual type III polyketide synthase (PKS) from Atropa belladonna, which condenses two malonyl-CoAs with NMPy in the absence of an additional starter unit to form 4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoic acid (MPOB), and a cytochrome P450, which catalyzes the oxidation and cyclization of MPOB to tropinone, thereby completing the biosynthetic pathway between arginine and tropine.

Although TA biosynthetic genes have been expressed in Escherichia coli and yeast for enzyme characterization or production of TAs from fed precursors, no microbial platforms have been engineered to produce medicinal TAs from central metabolites. Very recently, the production of tropine and pseudotropine in engineered S. cerevisiae was reported. However, limited optimization of precursor production in that study may present challenges in directing sufficient flux through the pathway to enable production of downstream TAs.

Here, we engineer a yeast platform for production of tropine from simple sugars. Our final strain combines four overexpressed yeast enzymes, seven disruptions to native regulatory proteins and biosynthetic enzymes, and nine heterologous genes: SPE1, CAR1, ARG2, and FMS1.

Results

Engineering a platform strain for putrescine overproduction. The tropine moiety of TAs is derived from arginine via putrescine, a polyamine required for ribosome biogenesis and mRNA translation. Putrescine and other polyamine concentrations are regulated to remain low during normal cell growth. We focused on engineering a putrescine-overproducing strain by overexpressing native genes involved in arginine metabolism and polyamine biosynthesis. Glutamate N-acetyltransferase (Arg2p) catalyzes the first step in arginine biosynthesis from glutamate. The guanidinium group of arginine is removed by an arginase (Car1p) to produce ornithine in the mitochondria, which is exported to the cytosol by an ornithine transporter (Ort1p). Cytosolic ornithine is decarboxylated to putrescine by an ornithine decarboxylase (ODC; Spe1p). Putrescine is also produced by dealkylation of spermine and spermidine by a polyamine oxidase (Fms1p).

To examine the effect of overexpression of these enzymes on putrescine production, we co-transformed wild-type yeast (CEN. PK2) with combinations of three low-copy plasmids, expressing SPE1 (pCS4211), ORT1 (pCS4194), CAR1 (pCS4195), ARG2 (pCS4196), FMS1 (pCS4199), or blue fluorescent protein (BFP; pCS4208, 4212, 4213) as a negative control. We quantified putrescine accumulation in the medium following 48 h of growth by LC-MS/MS (Fig. 2b). SPE1 overexpression resulted in a 13-fold increase in putrescine titer to 23 mg/L. While overexpression of CAR1 or ARG2 with SPE1 resulted in 28 and 13% increases in putrescine titer relative to SPE1 alone, overexpression of ORT1 with SPE1 caused a 35% decrease in putrescine. ORT1 overexpression caused impairment in growth rate, which may contribute to decreased putrescine production. Overexpression of any three of SPE1, CAR1, ARG2, and FMS1 increased putrescine titers to ~35 mg/L. The results suggest that a greater than 30-fold improvement in production of putrescine, a metabolite whose biosynthesis is regulated to remain at low levels (~1.5 mg/L), is achieved by overexpressing combinations of four genes: SPE1, CAR1, ARG2, and FMS1.
Module I: Arginine and polyamine metabolism

- Arginine
- Glutamic acid
- AsADC: Arg2p
- Car1p: Carbamoyltransferase
- SpeB: Agmatine deiminase
- Spe1p: Ornithine decarboxylase
- Fms1p: Polyamine oxidase
- N-Methylputrescine
- Ornithine
- Putrescine
- Spermine

Module II: Tropane alkaloid pathway

- Phenylalanine
- trans-Cinnamic acid
- Cinnamoyl-CoA

Module III: Phenylpropanoid pathway

- Cinnamoyl-CoA

Module IV: Acyl transfer

- EcCS: Erythroxylum coca cocaine synthase

Fig. 1 Engineered pathway for de novo biosynthesis of tropane alkaloids in yeast. Color scheme for enzyme labels: orange, overexpressed yeast enzymes; green, plant enzymes; violet, bacterial enzymes; grey, spontaneous (non-enzymatic) step. Enzyme symbols: Arg2p, glutamate N-acetyltransferase; Car1p, arginase; AsADC, Avena sativa arginine decarboxylase; SpeB, agmatine ureohydrolase; Spe1p, ornithine decarboxylase; Fms1p, polyamine oxidase; AbPMT1, Atropa belladonna putrescine N-methyltransferase 1; DmMPO1ΔC-PTS1, Datura metel N-methylputrescine oxidase 1 with peroxisome targeting sequence 1 and truncated C-terminus; AbPYKS, A. belladonna pyrrolidine ketide synthase; AbCYP82M3, A. belladonna tropinone synthase; AtATR1, Arabidopsis thaliana NADP⁺-cytochrome P450 reductase; AtPAL1, A. thaliana phenylalanine ammonia-lyase; At4CL5, A. thaliana 4-coumarate-CoA ligase 5; EcCS, Erythroxylum coca cocaine synthase.
We leveraged the diversity of polyamine pathways from other organisms to further increase putrescine production in yeast. These alternate biosynthetic routes differ in the order in which functional groups are removed from arginine (Fig. 2a). Many bacteria and plants express an alternate route through which arginine is decarboxylated by arginine decarboxylase (ADC) to agmatine. In plants, the guanidine group of agmatine is converted to a urea by an iminohydrolase (AIH) to produce N-carbamoylputrescine (NCP), from which the amide group is removed by an amidase (CPA) to yield putrescine. Some bacteria have an

arginase from
A. thaliana
SlCPA
A. thaliana
heterologous biosynthetic pathway from plants and bacteria. Heterologous enzymes were expressed from low-copy plasmids in wild-type yeast.

Putrescine pathways, indicated genes were expressed from low-copy plasmids in wild-type yeast (WT) or each single disruption strain.

For overexpression of native or heterologous putrescine pathways, indicated genes were expressed from low-copy plasmids in wild-type yeast (CEN.PK2).

Putrescine production in yeast strains with disruptions to endogenous polyamine biosynthesis regulatory mechanisms. For overexpression of the native and heterologous putrescine pathways, indicated genes were expressed from low-copy plasmids in wild-type yeast (WT) or each single disruption strain.

Although reports of AtARGAH2 exhibiting activity in S. cerevisiae,
A. sativa (AsADC with demonstrated activity in S. cerevisiae) and Arabidopsis thaliana (AtAIH), two CPA orthologs from Solanum lycopersicum (SlCPA) and A. thaliana (AtCPA), an AUH from E. coli (speB), and an arginase from A. thaliana (AtARGAH2) with demonstrated ureohydrolyase activity.

We reconstituted the three-step (arginine → agmatine → NCP → putrescine) or two-step (arginine → agmatine → putrescine) putrescine pathways in a stepwise fashion by co-transforming the wild-type strain with plasmids expressing AsADC (pCS4225), AtAIH (pCS4226), and either SlCPA (pCS4222) or AtCPA (pCS4221); or AsADC and any speB (pCS4223) or AtARGAH2 (pCS4224). All transformations were performed with three low-copy plasmids, using a BFP negative control plasmid (pCS4208, 4212, 4213). We analyzed relative accumulation of agmatine, NCP, and putrescine in the medium by LC-MS/MS after 48 h (Fig. 2c, Supplementary Fig. 2).

Reconstitution of the plant-specific pathway comprising AsADC, AtAIH, and AtCPA enabled putrescine titers of 23 mg/L, a 22-fold improvement relative to wild-type titers. SlCPA was poorly functional and enabled putrescine titers of 4.5 mg/L when combined with AsADC and AtAIH, similar to levels when expressing only AsADC and AtAIH. Reconstitution of the bacterial shortcut pathway via AsADC and SpeB enabled putrescine titers of 34 mg/L, 32-fold higher than wild-type. Despite reports of AtARGAH2 exhibiting activity in S. cerevisiae, we observed virtually no activity when this enzyme was co-expressed with AsADC.

We examined overexpression of the native pathway with the reconstituted plant-bacterial shortcut pathway to increase putrescine titers. We combined the top-performing triad of overexpressed native genes (SPE1, ARG2, CAR1) with the top-performing heterologous putrescine pathway (AsADC, speB) by co-transforming the wild-type strain with a low-copy plasmid encoding SPE1, AsADC, and speB (pCS4239) and low-copy plasmids encoding ARG2 (pCS4196) and CAR1 (pCS4216), and measured putrescine titers in the medium by LC-MS/MS after 48 h. The resulting strain (CSY1227) produced putrescine titers of 47 mg/L, greater than either pathway individually (CSY1225, CSY1226) but less than expected for a purely additive effect (Fig. 2d), indicating that polyamine regulatory mechanisms may be limiting putrescine production.

Yeast polyamine biosynthesis is regulated by several mechanisms (Fig. 2d). Methylthioadenosine phosphorylase (Meu1p) catalyzes the driving step in the recycling pathway for decarboxylated S-adenosylmethionine (dcSAM) from methylthioadenosine (MTA), which inhibits the activity of spermidine synthase (Spe3p)25. dcSAM-dependent alkylation of putrescine to spermidine and spermine is catalyzed by Spe3p and spermine synthase (Spe4p)30. We expected disruption of MEU1 and SPE4 to inhibit dcSAM-dependent alkylation of putrescine, reducing flux to other polyamines. Polyamine biosynthesis is also regulated by an antizyme-mediated negative feedback loop31, where the OAZ1 gene encodes antizyme-1, a competitive inhibitor of ornithine decarboxylase (Spe1p). A polyamine-induced ribosomal frameshift enables translation of full-length antizyme at high polyamine levels, thereby imposing feedback inhibition. We expected disruption of OAZ1 to eliminate this source of feedback inhibition limiting putrescine production. Finally, polyamine uptake is mediated by a signaling pathway involving Agp2p, a plasma membrane permease, and Sky1p, a protein kinase thought to interact with Agp2p and whose deletion reduces uptake of spermidine and spermine27,32. We expected disruption of AGP2 and SKY1 to reduce accumulation of spermidine and spermine, decreasing polyamine-induced expression of Oaz1p and alleviating Spe1p inhibition.

We examined deregulation of polyamine biosynthesis regulatory mechanisms to increase putrescine production by constructing single-gene disruptions for MEU1, OAZ1, SPE4, SKY1, and AGP2 by inserting nonsense mutations within each open reading frame in wild-type yeast. We overexpressed ODC (SPE1) or co-expressed AsADC and speB from low-copy plasmids (pCS4225, 4223) in each of the single-gene disruption strains, and measured putrescine titers in the medium via LC-MS/MS after 72 h (Fig. 2e). MEU1 and OAZ1 disruption improved putrescine titers by 68 and 174%, respectively, when the native pathway via SPE1 was overexpressed, but did not significantly impact titers when the heterologous AsADC/speB pathway was overexpressed, consistent with the mechanism of action for these enzymes. Disruption of OAZ1 resulted in a 21-fold increase in putrescine titer in cells with neither the native nor heterologous pathways overexpressed, highlighting the impact of the antizyme feedback inhibition system in restricting putrescine biosynthesis. Disruption of SKY1 and AGP2 resulted in 29 and 14% respective increases in putrescine titer when overexpressed with SPE1, but decreased titers by 41% with expression of AsADC and speB. Disruption of SPE4 did not impact putrescine production in the context of SPE1 overexpression or AsADC and speB expression. These results indicate that substantial improvements to putrescine production via the native Spe1p-dependent pathway may be achieved by disrupting MEU1 and OAZ1.

Finally, we combined the MEU1 and OAZ1 knockouts with overexpression of the native and heterologous putrescine biosynthetic genes. We integrated additional copies of the genes ARG2, CAR1, and FMS1 into the genome of a meu1/oaz1 double-disruption strain (CSY1234), and transformed this strain with a low-copy plasmid expressing SPE1, AsADC, and speB (pCS4239),...
resulting in strain CSY1235. LC-MS/MS analysis of the medium of CSY1235 indicated that putrescine titers reached 86 mg/L after 48 h (Fig. 2f), representing a 71-fold improvement relative to wild-type yeast.

Optimization of de novo N-methylpyrrolinium production. We proceeded with reconstitution of the subsequent biosynthetic steps towards tropine. The first two enzymes in the tropine pathway branch between putrescine and NMPy are common to nicotine biosynthesis33. Putrescine is converted to N-methylputrescine (NMP) by a SAM-dependent N-methyltransferase (PMT), which is oxidized to 4-methylaminobutanol (4MAB) by a copper-dependent diamine oxidase (MPO). 4MAB is unstable in aqueous solution and spontaneously cyclizes via intramolecular nucleophilic attack to form NMPy (Fig. 1).

We focused on optimizing NMPy production in our putrescine-overproducing strain. We co-transformed strain CSY1235, which harbors a low-copy plasmid expressing SPE1, AsADC, and speB (pCS4239), with low-copy plasmids expressing a PMT from A. belladonna (AbPMT1; pCS4193) and a MPO from Nicotiana tabacum (NtMPO1; pCS4218). All transformations were performed with a three-plasmid system as described above. We compared intermediate accumulation in the medium from cells expressing each successive enzyme between putrescine and NMPy by LC-MS/MS after 48 h. Although the product of NtMPO1 (4MAB) and its spontaneous cyclization product (NMPy) were detectable with expression of AbPMT1 and NtMPO1 (Fig. 3a), levels were far lower than their precursors, NMP and putrescine (Supplementary Fig. 3). We verified that MEU1 disruption and its impact on SAM recycling did not inhibit putrescine N-methylation by AbPMT1 (Supplementary Fig. 4).

We initially suspected that the low 4MAB/NMPy levels might be due to poor expression and/or activity of NtMPO1, possibly due to an unfavorable chemical environment resulting from incorrect subcellular localization. We explored improvements in MPO activity through a combination of subcellular localization studies, identification of MPO orthologs, and enzyme truncations (Supplementary Note 1). Although localization of MPO activity to the cytosol from the peroxisome (Supplementary Fig. 5b, Fig. 3b) did not yield improvement in NMPy production (Supplementary Fig. 5c), a C-terminally truncated MPO ortholog from Datura metel (DmMPO1AC-PTS1) resulted in 55 and 11% respective increases in 4MAB and NMPy titers relative to NtMPO1 (Supplementary Figure 6d, Fig. 3c). All further optimization was performed using DmMPO1AC-PTS1. 4MAB and NMPy levels remained lower than expected given the majority of NMP was consumed upon MPO expression (Fig. 3a, Supplementary Fig. 3), suggesting side reaction(s) that consume the product(s) of MPO, drawing flux away from TA biosynthesis. We observed accumulation of 4MAB acid by LC-MS/MS after 48 h when AbPMT1 and DmMPO1AC-PTS1 were co-expressed from low-copy plasmids (pCS4193, 4238) in the putrescine-overproducing strain (CSY1235), but no accumulation in the absence of MPO (Fig. 3a), indicating that aldehyde dehydrogenases may be oxidizing 4MAB.

We examined the role of six gene(s) (ALD2-ALD6, HDF1) encoding enzymes with aldehyde dehydrogenase activity34,35 in 4MAB acid production. ALD2 and ALD3 encode nearly identical cytosolic dehydrogenases, which catalyze the oxidation of 3-aminoipropanal to β-alanine36. ALD4, ALD5, and ALD6 encode mitochondrial and cytosolic acetaldehyde dehydrogenases, which oxidize acetaldehyde to acetate during fermentative growth37 and an array of aliphatic and aromatic aldehydes to carboxylic acids38. Based on the annotated dehydrogenase activity of HDF1 and ALD4-6 on 4-aminobutanol in the KEGG database, we constructed individual knockouts strains for these targets (CSY1236-1239) by inserting a series of nonsense mutations within their open reading frames in the putrescine-overproducing strain (CSY1235). We co-expressed AbPMT1 and DmMPO1AC-PTS1 from low-copy plasmids (pCS4193, 4238) in each single strain and measured 4MAB acid accumulation in the media by LC-MS/MS after 48 h. The HDF1 and ALD4-6 disruptions resulted in 13–27% decreases in 4MAB acid titer and marginal (<6%) increases in 4MAB or NMPy titer (Supplementary Fig. 7, Fig. 3d).

Although ALD4-6 are considered essential genes for their role in acetate and acetyl-CoA production32, the genes are partially redundant and the lethal phenotype of double and triple knockouts can be rescued by supplementing media with acetate37,38. We constructed a quadruple knockout strain (CSY1240) with disruptions to HDF1 and ALD4-6, and which expressed AbPMT1 and DmMPO1AC-PTS1 from low-copy plasmids (pCS4193, 4238). This strain showed a 34% reduction in 4MAB acid and 33 and 45% respective increases in 4MAB and NMPy titers compared to the strain with no disruptions (CSY1235) (Supplementary Fig. 7, Fig. 3d), indicating at least one other aldehyde dehydrogenase remained active on 4MAB. Although Ald2p and Ald3p do not oxidize the same diversity of substrates as Ald4p-Ald6p, the similarity of 4MAB to the NtMPO1 and ALD2-6 encoded nearly identical ALD2-6

Optimization of de novo tropeine biosynthesis in yeast. The precise mechanism and associated enzymes for the biosynthesis of tropine from NMPy remained unknown until recently, when a root-expressed PKS and cytochrome P450 were described in A. belladonna19. The identified PKS, pyrrolidine ketide synthase (PKS; DmPKS), uses the activated pyrrolinium moiety to initiate the first step of tropine biosynthesis, tropinone synthase (CYP82M3), catalyzes the condensation with two malonyl-CoA units to form MPOB. The synthase (PYKS), uses the activated pyrrolinium moiety to initiate the second ring closure to tropine18 (Fig. 4a). We obtained yeast codon-optimized DNA sequences encoding tropinone from NMPy. When a root-expressed PKS and cytochrome P450 were described in A. belladonna19. The identified PKS, pyrrolidine ketide synthase (PKS; DmPKS), uses the activated pyrrolinium moiety to initiate the second step of tropine biosynthesis, tropinone synthase (CYP82M3), catalyzes the condensation with two malonyl-CoA units to form MPOB. The P450 enzyme, tropinone synthase (CYP82M3), catalyzes the second ring closure to tropine by reforming the unsaturated pyrrolinium group via hydroxylation and subsequent dehydration19. Tropinone is reduced by a stereospecific 33,34. Based on the annotated dehydrogenase activity of HDF1 and ALD4-6 on 4-aminobutanol in the KEGG database, we constructed individual knockouts strains for these targets (CSY1236-1239) by inserting a series of nonsense mutations within their open reading frames in the putrescine-overproducing strain (CSY1235). We co-expressed AbPMT1 and DmMPO1AC-PTS1 from low-copy plasmids (pCS4193, 4238) in each single strain and measured 4MAB acid accumulation in the media by LC-MS/MS after 48 h. The HDF1 and ALD4-6 disruptions resulted in 13–27% decreases in 4MAB acid titer and marginal (<6%) increases in 4MAB or NMPy titer (Supplementary Fig. 7, Fig. 3d).

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We obtained yeast codon-optimized DNA sequences encoding A. belladonna pyrrolidine ketide synthase (AbPKYS), tropinone synthase (AbCYP82M3), Datura stramonium tropinone reductase I (DsTR1), and four different CPRs, including plant CPRs from A. thaliana, Eschscholzia californica, and Papaver somniferum and the yeast CPR (YCP1). We constructed strain CSY1246 by
integrating DsTR1 into the genome of the NMPy-producing strain (CSY1243), and expressing AbPYKS (pCS4246), AbCYP82M3 (pCS4247), and each CPR (pCS4200-4203) from low-copy plasmids. We monitored accumulation of NMPy, MPOB, tropinone, and tropine by LC-MS/MS in the media after 48 h (Fig. 4b, Supplementary Fig. 9b). Tropine production levels were comparable (175–210 μg/L) with all CPR partners. Although accumulation of tropinone, the product of AbCYP82M3, was...
minimal, a substantial portion of MPOB remained unconsumed by AbCYP82M3 (Supplementary Fig. 9b), implicating the P450 enzyme as a primary bottleneck (Supplementary Discussion). In addition, hygrine, a derivative of NMPy, accumulated to titers almost four-fold greater than tropine (775–900 µg/L). Hygrine accumulation had previously been observed during in vitro characterization of AbPYKS and AbCYP82M3 via spontaneous decarboxylation of MPOB19 (Fig. 4a). We observed reduced hygrine accumulation in a control strain lacking AbPYKS and AbCYP82M3, suggesting a second mechanism for hygrine production in the yeast environment. We hypothesized that a metabolite present in the media and/or produced by yeast might undergo spontaneous decarboxylative condensation with NMPy to form hygrine (Fig. 4a). We performed co-substrate feeding experiments with NMPy-producing and -non-producing strains, which suggested that acetate supplementation may increase levels of an endogenous keto-metabolite, such as acetoacetate or acetone, which suggested that acetate supplementation may increase levels of an endogenous keto-metabolite, such as acetoacetate or acetone, which suggested that acetate supplementation may increase levels of an endogenous keto-metabolite, such as acetoacetate or acetone, which suggested that acetate supplementation may increase levels of an endogenous keto-metabolite, such as acetoacetate or acetone, which suggested that acetate supplementation may increase levels of an endogenous keto-metabolite, such as acetoacetate or acetone, which suggested that acetate supplementation may increase levels of an endogenous keto-metabolite, such as acetoacetate or acetone, which suggested that acetate supplementation may increase levels of an endogenous keto-metabolite, such as acetoacetate or acetone, which suggested that acetate supplementation may increase levels of an endogenous keto-metabolite, such as acetoacetate or acetone, which suggested that acetate supplementation may increase levels of an endogenous keto-metabolite, such as acetoacetate or acetone, which suggested that acetate supplementation may increase levels of an endogenous keto-metabolite, such as acetoacetate or acetone, which suggested that acetate supplementation may increase levels of an endogenous keto-metabolite, such as acetoacetate or acetone. Plasmid-based overexpression of eukaryotic P450s can impose a significant burden on yeast cells due to over-proliferation of ER membranes29. We examined integration of the tropine biosynthesis genes into the yeast genome to enable more stable AbCYP82M3 expression and improve tropine production. Given the ability of the A. thaliana CPR (AtATR1) to pair with various plant P450 enzymes3, we constructed a tropine-producing platform strain (CSY1248) by integrating AtATR1, AbPYKS, and AbCYP82M3 into the genome of CSY1246. We compared tropine and hygrine accumulation for CSY1248 to plasmid-based expression (pCS4236, 4237, 4238) in CSY1246 together with SPE, AsADC, and speB (pCS4239) and AbPYKS (pCS4193). We attempted to reduce hygrine production from spontaneous condensation with an endogenous acetate-derived metabolite by eliminating acetate auxotrophy. We observed that reconstitution of ALD6 in CSY1249 resulted in a 2.7-fold increase in tropine titers (1.5 mg/L) relative to CSY1248 (565 µg/L) despite a 1.6-fold increase in hygrine accumulation (Fig. 4d), and that elimination of acetate auxotrophy may improve metabolite flux through the entire pathway (Supplementary Note 3).

Optimization of flux bottlenecks and media conditions. To identify pathway bottlenecks between putrescine and tropine, we expressed an additional copy of AbPMT1 (pCS4193), DmMOPI1-Δc-PTS1 (pCS4238), AbPYKS (pCS4246), AbCYP82M3 (pCS4247), or DsTR1 (pCS4310) from low-copy plasmids in CSY1249 and compared intermediate production to that of CSY1249 expressing BFP (pCS4208, pCS4212, or pCS4213) by LC-MS/MS analysis. All data represent the mean of n = 3 biologically independent samples (open circles in c, d) and error bars show standard deviation. Student’s two-tailed t-test: *P < 0.05, **P < 0.01, ***P < 0.001. Unless otherwise indicated, statistical significance is shown relative to the corresponding control (CSY1235). Source data of Fig. 3c and d are provided as a source data file.
low-density cultures (LDCs), wherein initial cell density is low (OD ~ 0.1–0.5), and high-density cultures (HDCs) in which cells are grown to a moderate initial density (OD ~ 1.5) and transferred to fresh media. We approximated a fed-batch culture by supplementing media with dextrose, glycerol, and amino acids after three days. Tropine titers reached 5.9 mg/L in HDC (ODfinal ~ 2.8) and 2.2 mg/L in LDC (ODfinal ~ 1.9), highlighting the importance of starting culture density (Fig. 4f). Although NMP and tropinone did not accumulate appreciably, titers of hygrine, putrescine, and NMPy reached 10, 30, and 30 mg/L, respectively, in HDC (Supplementary Fig. 14). Overall, our strategies for optimizing tropine production resulted in a 28-fold improvement in titer to nearly 6 mg/L.

De novo production of the non-canonical TA cinnamoyltropine. Medicinally relevant TAs such as hyoscyamine and scopolamine are derived from littorine, the product of tropine condensation with phenyllactate. Although the enzymes catalyzing this reaction have not yet been identified in Solanaceae, analogous TAs and associated acyltransferases have been...
identified in *Erythroxylaceae*, an evolutionarily distant lineage of plants which have thought to have evolved the ability to produce TAs independently. An acyltransferase denoted caffeoyl synthase from *Erythroxylum coca* (EcCS) catalyzes condensation of caffeoyl-CoA (a coenzyme A (CoA)-activated acyl donor, we used a corresponding to the parent mass of cinnamoyltropine, in the medium via α and measured accumulation of the product, cinnamoyl-3 tropine (referred to as cinnamoyltropine), in the medium via LC-MS/MS analysis. Cinnamate donors when expressed in yeast, including metabolites from plant phenylpropanoid pathways. We used our engineered yeast platform to combine TA biosynthetic genes from *Solanaceae* and *Erythroxylaceae* with an acyl donor biosynthetic module to enable de novo production of TAs.

We selected cinnamic acid, a phenylpropanoid intermediate whose biosynthesis in yeast has been demonstrated, as the acyl donor for condensation with tropine by EcCS (Fig. 1). Cinnamate can be produced from phenylalanine via a phenylalanine ammonia-lyase from *A. thaliana* (AtPAL1). Since EcCS requires a coenzyme A (CoA)-activated acyl donor, we used a 4-coumarate-CoA ligase from *A. thaliana* (At4CL5) with activity on cinnamate to enable cinnamoyl-CoA biosynthesis. We obtained yeast codon-optimized DNA sequences for these genes and assembled them into a two-plasmid expression system. AtPAL1 was expressed from a low-copy plasmid (pCS4252), whereas At4CL5 and EcCS were expressed from a high-copy plasmid (pCS4207) to compensate for the low activity of EcCS on non-native substrates. We expressed AtPAL1, At4CL5, and EcCS in our tropine-producing strain (CSY1251), denoted CSY1282, and measured accumulation of the product, cinnamoyl-3α-tropine (referred to as cinnamoyltropine), in the medium via LC-MS/MS after 72 h.

We used tandem MS/MS and fragmentation analysis to verify the identity of this product. Comparison of MS/MS spectra corresponding to the parent mass of cinnamoyltropine (m/z = 272) revealed a peak at a retention time of 3.684 min for CSY1282 but not for CSY1251, which produced fragments whose retention time and mass transitions were identical to those generated by a cinnamoyl-3α-tropine standard (Fig. 5a). The primary m/z = 272 → 124 transition, which is consistent with the m/z = 124 tropine fragment produced during fragmentation of hyoscyamine, was used to develop a multiple reaction monitoring (MRM) LC-MS/MS method and standard curve for quantification of cinnamoyltropine. The titer of cinnamoyltropine produced by CSY1282 after 72 h was 6.0 μg/L. We performed substrate feeding experiments in tropine-producing and non-producing strains to elucidate the stereochemistry of the EcCS-catalyzed reaction (Supplementary Note 5, Fig. 5b–i).

**Discussion**

Although several groups have engineered prokaryotes for polyamine overproduction, few such efforts have been made in eukaryotic hosts. An earlier study engineered *S. cerevisiae* to overproduce spermidine based on disruption of *OAZ1* and *TPO1*, resulting in a 2.7-fold increase in spermidine titers. The 71-fold increase in putrescine titers observed here highlights the importance of our multi-target strategy for increasing polyamine accumulation. Although subsequent experiments (Supplementary Figs. 8, 12, 14) indicated that putrescine production was not limiting flux, future efforts for improving titers towards industrial TA production may incorporate more comprehensive alterations to nitrogen metabolism, such as partial bypass of the urea cycle or upregulation of arginine and ornithine biosynthesis via pathway rewiring and flux balancing.

Ping et al. recently reported production of NMPy in *E. coli* and *S. cerevisiae* strains. Expression of three heterologous genes—an ODC ortholog from *E. coca* and PMT and MPO variants from *Antisodus* species-enabled NMPy production at nearly 20 mg/L in yeast. Putrescine accumulation due to the heterologous ODC may have inhibited the native ODC through the antizyme-mediated feedback regulation, resulting in reduced putrescine titers (15 mg/L) relative to that achieved in our platform (86 mg/L). Consistent with our observations of 4MAB oxidation by aldehyde dehydrogenases, the prior study noted Ald4p, Ald5p, and Hfd1p convert 4MAB to 4MAB acid in vitro and deleted these genes to improve NMPy accumulation. Our work shows that disruption of Hfd1p and Ald4p–Ald6p activity eliminates less than half of the 4MAB acid, and additional disruption of Ald2p and Ald3p activity eliminates this side product. Although the possibility that Hfd1p and Ald2p–Ald6p function synergistically to oxidize 4MAB prevents drawing quantitative conclusions regarding individual enzyme contributions to this side product, our work indicates that disruption of *HFD1* and *ALD2-6* is essential for achieving high NMPy production.

Although our optimization of putrescine production enabled NMPy titers (40 mg/L) roughly two-fold greater than previously reported, the conversion of putrescine to NMPy was less efficient. Data for the NMPy-producing strain (CSY1243) indicated that despite near complete consumption of putrescine and NMP, NMPy accumulated to less than half the titer observed for putrescine in CSY1235 (>80 mg/L), suggesting a leak in metabolite flux between these TA precursors. We hypothesize that some putrescine may be converted to other polyamines or amino acids due to inefficient N-methylation by AbPMT1 and/or oxidation by DmMPO1. TA biosynthesis occurs in roots,
whereas the DmMPO1 variant was identified from a leaf transcriptome library and thus may not efficiently utilize NMP. Replacement of DmMPO1 with a root-expressed variant with higher catalytic efficiency and improving SAM availability for the methyltransferase step may increase flux from putrescine to NMPy.

Hygrine constitutes a major side product in the biosynthesis of tropine in yeast, corroborating a prior study that observed substantial hygrine accumulation during in planta and in vitro characterization of AbPYKS and AbCYP82M3. While the study noted accumulation of cuscohygrine in planta and in vitro due to degradative condensation of an additional NMPy unit with MPOB, we were unable to observe this side product in yeast, highlighting the utility of microbial hosts for the production of plant natural products with improved selectivity. Our work suggests that hygrine production in yeast likely occurs via two mechanisms: (i) spontaneous decarboxylation of MPOB, and (ii) spontaneous condensation of NMPy with keto-metabolites. Although we reduced the contribution of the second mechanism by reconstituting ALD6 to abrogate acetate auxotrophy and the contributions of both mechanisms by lowering temperature, the decrease in hygrine production was overshadowed by the positive effect of ALD6 on pathway flux. The generation of NADPH through dehydrogenase activity of ALD6 likely provided a more kinetically favorable NADPH:NADP+ ratio for activity of the cytochrome P450/CPR pairing and NADPH-dependent TRI, similar to the effect of glycerol supplementation (Supplementary Note 4). Future strategies for improving flux may reduce hygrine production from spontaneous MPOB decarboxylation. For example, colocalizing AbPYKS and AbCYP82M3 may reduce latency between MPOB production and oxidation. A similar colocalization strategy may be applied to enzymes involved in NMPy biosynthesis to decrease condensation of NMPy with keto-metabolites.

Although our bottleneck analysis revealed that the PMT and PYKS steps were limiting tropine flux, time course data for the final tropine-producing strain (CSY1251) revealed that key inefficiencies remain. Approximately 30 mg/L of putrescine and NMPy accumulated (Supplementary Fig. 14), indicating that low flux of putrescine into NMPy and NMPy into tropine remain major bottlenecks. We inferred that some aspect of the PKS enzyme other than expression might be limiting, such as malonyl-CoA availability or poor inherent activity in yeast. Incorporation of strategies to increase malonyl-CoA availability or and to engineer PYKS for improved catalytic efficiency may increase tropine production.

While this manuscript was under final review, Ping et al. reported the biosynthesis of tropine in yeast by expressing A. acutangulus PYKS, CYP82M3, and TRI orthologs in a NMPy-producing strain. Little optimization of pathway flux from NMPy to tropine was beyond expression of an additional copy of native ACC1 to improve malonyl-CoA availability. Through extensive optimization of precursor production, enzyme expression, pathway bottlenecks, side reactions, and growth conditions, we achieved tropine titers (5.9 mg/L) nearly 50-fold
greater than those reported in that study (0.13 mg/L), providing a robust starting point for downstream TA biosynthesis. Although many of the enzymes required for biosynthesis of medicinally important TAs, such as hyoscyamine and scopolamine, are now identified, key gaps still remain. Our tropine-producing strain offers a platform for rapidly screening and testing gene candidates for missing enzyme activities, enabling more accessible and cost-effective production of these important medicines. Tropinone and tropine are precursors for a variety of alkaloids, including calyctegines and derivatives of cocaine. Extension of the engineered pathway to these compounds will facilitate characterization of their associated enzymes and study of their biological applications. Finally, as demonstrated with biosynthesis of cinnamoyltropine, coupling biosynthetic modules from diverse plant lineages to produce different acyl donors and acceptors within a heterologous host enables generation of new non-canonical or non-natural TA derivatives, thereby accelerating the drug discovery pipeline.

Methods

Chemical compounds and standards. Putrescine dihydrochloride, N-methylpyr- tressine, hygrine, tropinone, and tropine were purchased from Santa Cruz Bio-
techology (Dallas, TX). 4-(Methylamino)butyric acid hydrochloride, lithium acetate, and cinchonic acid were purchased from Sigma (St. Louis, MO).

Pseudotropine was purchased from Chem-Impex International (Wood Dale, IL). γ-Methylaminobutyraldehyde (4MAB) diethyl acetal was purchased from Toronto Research Chemicals (Toronto, ON). 4MAB was prepared from 4MAB diethyl acetal by treatment with an equal volume of 2 M HCl for 30 min at 60 °C. NMPy was prepared from 4MAB diethyl acetal by treatment with five volumes of 2 M HCl for 30 min at 60 °C, overnight incubation at room temperature, washing with three volumes of ether to remove trace organic impurities, and evaporation of residual water under vacuum. Cinnamoyltropine (catalogue number K23.206.020) was synthesized by Aurora Fine Chemicals LLC (San Diego, CA).

NMR verification of cinnamoyl-3α-tropine standard. To verify the identity and stereochernistry of the synthesized cinnamoyltropine as the ester of trans-cinnamic acid and tropine, 1H-NMR was performed on the standard as well as on purchased standards of trans-cinnamic acid, tropine, and pseudotropine using CD3OD as the solvent on a Varian Inova 600 MHz spectrometer at the Stanford University NMR Facility (Stanford, CA). Spectrum analysis and processing was performed using the Mnova software package (Mestrelab Research, v12.0.4). 1H-NMR spectra are provided in Supplementary Figs. 15–18.

NMR data of cinnamoyltropine were as follows: δ 7.72 (d, J = 16.0 Hz, 1 H), 7.64 (m, 2 H), 7.43 (m, 3 H), 6.58 (d, J = 16.1, 14.4 Hz, 1 H), 5.36 (d, J = 15.3 Hz, 2 H), 2.81 (s, 3 H), 2.45 (m, 4 H), 2.35 (m, 2 H), 2.19 (m, 2 H), 1.92 (m, 2 H).

NMR data of trans-cinnamic acid were as follows: δ 17.0 MHz, CD3OD) δ 7.72 (d, J = 16.0 Hz, 1 H), 7.64 (m, 2 H), 7.43 (m, 3 H), 6.58 (d, J = 16.1, 14.4 Hz, 1 H), 5.36 (d, J = 15.3 Hz, 2 H), 2.81 (s, 3 H), 2.45 (m, 4 H), 2.35 (m, 2 H), 2.19 (m, 2 H), 1.92 (m, 2 H).

Plasmid construction. Oligonucleotides were used in this work were synthesized by the Stanford Protein and Nucleic Acid Facility (Stanford, CA) and are listed in Supplementary Data 1. Biosynthetic genes used in this study are listed by source and accession number in Supplementary Table 1. Native yeast genes were amplified from S. cerevisiae genomic DNA via colony PCR. Gene sequences for heterologous enzymes were codon-optimized to improve expression in S. cerevisiae using GeneGener8r (Integrated DNA Technologies, IDT) or thermostable scientific primer and synthesized as either gBlock DNA fragments (Integrated DNA Technologies, IDT) or gene fragments (Twist Bioscience). Plasmids used in this study are listed in Supplementary Data 2. Two types of plasmids were used for gene expression in yeast: direct expression (DE) plasmids for testing biosynthetic genes of interest and yeast integration (YI) plasmids for genomic integration of selected promoter-gene-terminator cassettes.

DE plasmids comprised a gene of interest flanked by a constitutive promoter and terminator, a low-copy CEN/ARS yeast origin of replication, and an auxotrophic selection marker. DE plasmids were constructed by PCR-amplifying gene of interest with 5′ and 3′ restriction sites using primer overhangs (Supplementary Data 1), digesting PCR products or synthesized gene fragments with appropriate pairs of restriction enzymes (Ssp, BamHI, EcoRI, PstI, and/or XhoI), and then ligating gene fragments into similarly digested vectors pAG414GPD-ccdB, pAG415GPD-ccdB, or pAG16GPD-ccdB using T4 DNA ligase, as detailed in Supplementary Data 1. YI plasmids comprised a gene of interest flanked by a constitutive promoter and terminator but lacked a yeast origin of replication or auxotrophic selection marker. YI plasmids were constructed by linearizing empty holding vectors pCS2656, pCS2657, pCS2658, pCS2661, or pCS2663 using around-the-horn PCR with primers designed to bind to the 5′ and 3′ ends of the promoter and terminator, respectively (Supplementary Data 1). Genes of interest were PCR-amplified to append 5′ and 3′ overhangs with 35–40 bp of homology to the termini of the linearized vector backbones. Assembly of genes into YI vectors was performed using assembly PCR, DE plasmids were used as fragments prepared by PCR-amplified DNA fragments separately encoding GFP, the target enzyme, and a YI vector backbone using Gibson assembly, and subsequently subcloning the fusion constructs from YI plasmids into DE vectors using restriction enzymes and ligation cloning.

All PCR amplification was performed using Q5 DNA polymerase (NEB) and linear DNA was purified using the DNA Clean and Concentrator-5 kit (Zymo Research). Assembled plasmids were propagated in chemically competent E. coli (TOP10, Thermo Fisher Scientific) using heat-shock transformation and selection in Luria-Bertani (LB) broth or on LB-agar plates with either carbenicillin (100 μg/mL) or kanamycin (50 μg/mL) selection. Plasmid DNA was purified by alkaline lysis from overnight E. coli cultures grown at 37 °C and 250 rpm in selective LB media using Ecospin columns (Epoch Life Science) according to the manufacturer’s protocol. Plasmid sequences were verified by Sanger sequencing (Quintara Biosciences).

Yeast strain construction. Strains used in this work (Supplementary Table 2) were derived from the parental strain CEN.PK-1D58, referred to as CEN.PK2. Strains were directly plated onto YNB(A)-DO agar plates. For Cas9-mediated chromosomal modifications, integration fragments comprised up to 8–10 restriction sites flanked by unique promoters and terminators were constructed using PCR amplification and cloned into holding vectors by Gibson assembly as described in Plasmid construction (Supplementary Fig. 1). Integration fragments were PCR-amplified using Q5 DNA polymerase (NEB) with flanking 40 bp microhomology regions to adjacent fragments and/or to the yeast genome at the integration site (Supplementary Data 1). For gene disruptions, integration fragments comprised 6–8 stop codons in all three reading frames flanked by 40 bp of microhomology to the targeted integration site, which collapsed into the flanking frame. For complete gene deletions, integration fragments comprised an auxotrophic marker flanked by 40 bp of microhomology to the deletion site. Approximately 300 ng of each integration fragment was co-transformed with 300 ng of CRISPRm plasmid targeting the desired genomic site. Positive integrants were identified by yeast colony PCR, Sanger sequencing, and/or functional screening by liquid chromatography and tandem mass spectrometry (LC-MS/MS).

Yeast transformations. Yeast strains were chemically transformed using the Frozen-EZ Yeast Transformation II Kit (Zymo Research). Individual colonies were inoculated into YP(A)D media and grown overnight at 30 °C and 250 rpm. Saturated cultures were back-diluted between 1:10 and 1:50 in YP(A)D media and grown for an additional 5–7 h to reach exponential phase. Cultures were pelleted by centrifugation at 500 × g for 4 min, then washed once with the pellet in 50 mM Tris-HCl buffer, pH 8.5. Washed pellets were resuspended in 20 μL of E22 solution per transformation and then mixed with 100–600 ng of total DNA and 200 μL of E23 solution. The yeast suspensions were incubated at 30 °C with gentle rotation for one hour. For plasmid transformations, the transformed yeast were directly plated onto YNB(–A)-DO agar plates. For Cas9-mediated chromosomal modifications, yeast suspensions were instead mixed with 1 mL YP(A)D media, pelleted by centrifugation at 500 × g for 4 min, and then resuspended in 250 μL of fresh YP(A)D media. The suspensions were incubated at 30 °C with gentle rotation for an additional two hours to enable production of G418 resistance plateaux (50 μg/mL kanamycin). For CRISPRm transformations, yeast suspensions were immediately plated onto YNB(–A)-DO agar plates and incubated at 30 °C for 48–72 h before being used to inoculate cultures for metabolite assays.
Spot dilution assays. Strains were inoculated into YNB(A)-DO media and grown overnight at 30 °C and 250 rpm. Saturated overnight cultures were pelleted by centrifugation at 500 × g for 4 min and resuspended in sterile Tris-HCl buffer, pH 8.0 to a concentration of 10^7 cells/mL based on OD_{600}. Ten-fold serial dilutions of each strain were prepared in Tris-HCl buffer and 10 μL of each dilution was spotted on pre-warmed YNB(A)-DO plates. Plates were incubated at 30 °C and imaged after 48 h.

Growth conditions for metabolite assays. Small-scale metabolite production tests were conducted in YNB(A)-SC or YNB(A)-DO media at in at least three replicates. Yeast colonies were inoculated into 300 μL of media and grown in 2 mL deep-well 96-well plates covered with AeraSeal gas-permeable film (Excel Scientific). Unless otherwise specified, cultures were grown for 48 h at 30 °C, 460 rpm, and 80% relative humidity in a Lab-Therm LX-T shaker (Adolf Kuhner).

Growth conditions for media optimization. Strains were inoculated in triplicate into YPD media and grown overnight to saturation at 30 °C and 250 rpm in glass culture tubes. After 18 h of growth, cultures were back-diluted 10× into 300 μL of YNB-SC or YP media supplemented with corresponding carbon sources and grown in 2 mL deep-well 96-well plates covered with AeraSeal gas-permeable film (Excel Scientific) in triplicates for 72 h at 25 °C, 460 rpm, and 80% relative humidity in a Lab-Therm LX-T shaker (Adolf Kuhner).

Growth conditions for time courses. For conventional low-density batch cultures, strains were inoculated in triplicate into YP(A)D media and grown overnight to saturation at 30 °C and 250 rpm. Overnight cultures were back-diluted to OD_{600} = 0.5 (for acetate-auxotrophic strains) or 0.1 (for acetate-prototrophic strains) in YNB(A)-SC media supplemented with appropriate carbon sources and grown in 50-mL shake flasks with 10 mL starting volume in triplicates at 30 °C or 25 °C and 300 rpm for 96–144 h. To simulate high-density batch culture conditions, strains were inoculated in triplicate into 10 mL of YPD media and grown overnight to saturation at 30 °C and 250 rpm. Saturated cultures were pelleted by centrifugation at 500 × g for 4 min and 3000 × g for 1 min and then resuspended in 10 mL of fresh YNB-SC or YP media supplemented with appropriate carbon sources and grown in 50-mL shake flasks with 10 mL starting volume in triplicates at 25 °C and 300 rpm for 144 h. Where indicated, fed-batch conditions were approximated by supplementing cultures after 72 h of growth with appropriate carbon sources and amino acids at 1× and 1× final concentrations, respectively. At appropriate time points, 250 μL samples were removed from cultures for analysis; 100 μL of culture was diluted 10× for optical density measurement and 0.4 mL of culture was used for metabolite quantification.

Analysis of metabolite production. Cultures were pelleted by centrifugation at 3500 × g for 5 min at 12 °C and 100–200 μL aliquots of the supernatant were removed for direct analysis. Metabolite production was analyzed by LC-MS/MS using an Agilent 1200 Infinity Binary HPLC and an Agilent 6420 Triple Quadrupole mass spectrometer. Chromatography was performed using a Zorbax EclipsePlus C18 column (2.1 × 50 mm), 1.8 μm; Agilent Technologies) with 0.1% formic acid and water 30/70 as solvent A and 0.1% formic acid in acetonitrile as solvent B. The column was operated with a constant flow rate of 0.4 mL/min at 40 °C and a sample injection volume of 5 μL. Compound separation was performed using the following gradient: 50.0%–0.75 min, 1% B; 0.75%–1.33 min, 1–23% B; 1.33–2.70 min, 23–40% B; 2.70–3.70 min, 40–60% B; 3.70–3.71 min, 60–95% B; 3.71–4.33 min, 95% B; 4.33–4.34 min, 95–1% B; 4.34–5.00 min, equilibration with 1% B. The LC eluent was directed to the MS from 0.01–5 min operating with electrospray ionization (ESI) in positive mode, source gas temperature 350 °C, gas flow rate 11 L/min, and nebulizer pressure 40 psi. Metabolites were quantified by integrated peak area in MassHunter Workstation software (Agilent) based on the multiple reaction monitoring (MRM) parameters in Supplementary Table 3 and standard curves (Supplementary Fig. 19). Primary MRM transitions were identified by analysis of 0.1–1 mM aqueous standards using the MassHunter Optimizer software package (Agilent) and corroborated against published mass transitions if available, and/or against predicted transitions determined using the CDF-1D fragment prediction utility54 and the METLIN database48.

Fluorescence microscopy. Individual colonies of yeast strains transformed with plasmids encoding biosynthetic enzymes fused to fluorescent protein reporters were inoculated into 1 mL YNB-DO media and grown overnight at 30 °C and 250 rpm. Overnight cultures were pelleted by centrifugation at 500 × g for 4 min and resuspended in 2 mL YNB-DO media with 2% w/v dextrose and then grown in 30 °C and 250 rpm for an additional 4–6 h to reach exponential phase and allow expressed fluorescent proteins to fold completely. Approximately 5–10 μL of culture was spotted onto a glass microscope slide and covered with a glass coverslip (Thermo Fisher Scientific) and then imaged using a Nikon TE2000 inverted microscope with a ×60 oil immersion objective. Fluorescence microscopy was performed using a LambdaXl xenon arc lamp (Sutter Instrument Company) and the following filter settings: GFP, ET470/40X excitation filter and ET525/50 emission filter; mCherry, ET572/35X excitation filter and ET632/60 emission filter. Emitted light was captured with a CoolSNAP HQ2 CCD camera (Photometric Scientific) and Micro-Manager software, and subsequent image analysis was performed in ImageJ (NIH). Images were converted to pseudocolor using the ‘Merge Channels’ and ‘Split Channels’ functions in ImageJ (Image → Color → Merge/Split Channels). Histogram stretching was applied equally across all images for a given channel (bright field, GFP, or mCherry) to improve contrast.

Identification of orthologs from transcriptome databases. Orthologs of N. tabacum N-methylputrescine oxidase (NtMPO1) were identified using a BLAST search of the transcriptomes of D. metel and A. belladonna in the 1000 Plants Project database55. This search yielded only one unique sequence from each of the two transcriptomes that was full-length (i.e., within 10% of the length of the query sequence, 790 residues) and with expectation value 0.0: JNVS_scaffold_2009311 (D. metel) and BOLZ_scaffold_2171654 (A. belladonna). Coding sequences for these two putative genes were optimized for yeast expression and then cloned into expression vectors as described in the section on plasmid construction above.

Enzyme structural analysis via homology modeling. Heterologous enzymes were analyzed for structural features that may prove problematic during expression in yeast, such as large unstructured regions, by examining homology models constructed using RaptorX with default modeling parameters56. Resultant protein models were visualized using PyMOL (Schrodinger).

Statistics. Where indicated, the statistical significance of any differences in metabolite titer between conditions was verified using Student’s two-tailed t-test. Biological replicates are defined as independent cultures inoculated from separate yeast colonies or streaks and cultivated in separate containers.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Data supporting the findings of this work are available within the paper and its Supplementary Information files. A reporting summary for this Article is available as a Supplementary Information file. The datasets generated and analyzed during the current study are available from the corresponding author upon request. The source data underlying Figs. 2b, c, e–f, 3c, d, and 4b–f, as well as Supplementary Figs. 2, 4, 5c, 6b, d, 7, 8, 9b, 11b, c, 12, 13, 14, and 19 are provided as a Source Data file.

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Author contributions
P.S. and C.D.S. conceived of the project, designed the experiments, analyzed the results, and wrote the manuscript. P.S. performed the experiments.

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Competing interests: The authors declare the following competing interests: P.S. and C.D.S. are inventors on a pending patent application; C.D.S. is a founder and CEO of Antheia, Inc.

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