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# Engineered Biosynthesis of Non-Natural Alkaloids via Modular P450 Engineering

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**[Abstract]**

**Alkaloids represent a vast class of natural products with significant pharmaceutical value, yet their structural complexity often hinders scalable production and diversification. Cytochrome P450 enzymes play pivotal roles in alkaloid biosynthesis, catalyzing regioselective and stereoselective oxidations. Here, we report a modular P450 engineering strategy to produce non-natural alkaloids in *Saccharomyces cerevisiae*. By combining directed evolution and rational design, we generated chimeric P450s with altered substrate specificity and enhanced catalytic efficiency. A library of 24 engineered P450 variants was constructed and screened against a panel of 12 non-natural alkaloid precursors. Three variants, designated P450-7, P450-12, and P450-19, exhibited high activity toward precursors bearing halogen and methyl substitutions. The best variant, P450-12, showed a 15-fold improvement in turnover number compared to the wild-type enzyme. Pathway optimization enabled de novo biosynthesis of 6-fluororetinine and 8-methylcorydaline at titers of 45 mg/L and 32 mg/L, respectively. Our results demonstrate that modular P450 engineering can expand the biosynthetic repertoire to access non-natural alkaloids, providing a platform for drug discovery and metabolic engineering.**

## Introduction

Alkaloids are nitrogen-containing natural products that exhibit a wide range of pharmacological activities, including analgesic, anticancer, and antimicrobial properties (Herbert, 1986, 1987, 1991). The structural diversity of alkaloids arises from complex biosynthetic pathways involving multiple enzyme families, among which cytochrome P450 monooxygenases (P450s) are particularly prominent (Podust & Sherman, 2012). P450s catalyze regio- and stereoselective hydroxylations, epoxidations, and C–C bond formations, often serving as gatekeepers for downstream tailoring reactions (Kishimoto et al., 2016; Sawai & Saito, 2011). Despite their catalytic prowess, the application of P450s in the biosynthesis of non-natural alkaloids—compounds with unnatural substituents or backbones—remains underexplored.

Recent advances in synthetic biology and metabolic engineering have enabled the heterologous production of several alkaloid classes in microbial hosts, such as benzylisoquinoline alkaloids (Menéndez-Perdomo & Facchini, 2018), tropane alkaloids (Srinivasan & Smolke, 2020), and ephedra-type alkaloids (Wu et al., 2024). However, the introduction of non-natural functional groups, such as halogens or alkyl chains, poses challenges due to the strict substrate specificity of native P450s (Amrine et al., 2019). To address this, we adopted a modular P450 engineering approach, combining directed evolution and rational design to create chimeric enzymes with expanded substrate scope. We hypothesized that by swapping substrate recognition domains between P450s from different alkaloid pathways, we could generate variants capable of oxidizing non-natural precursors. This strategy leverages the modular nature of P450s, as previously exploited in the biosynthesis of non-natural polyketides (Doekel & Marahiel, 2001) and terpenoids (Bohlmann & Keeling, 2008).

Here, we report the construction and characterization of a library of 24 engineered P450 variants, their screening against 12 non-natural alkaloid precursors, and the optimization of de novo biosynthetic pathways in *Saccharomyces cerevisiae* for the production of two non-natural alkaloids: 6-fluororetinine and 8-methylcorydaline. Our findings demonstrate that modular P450 engineering can effectively expand the biosynthetic repertoire, enabling access to structurally novel alkaloids for drug discovery.

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## Literature Review

The biosynthesis of alkaloids has been extensively studied in plants and microorganisms, with numerous pathways elucidated at the genetic and enzymatic levels (Herbert, 1986, 1987, 1991; Kato & Ashihara, 2008; Wang & Chen, 2014). P450s are ubiquitous in these pathways, catalyzing key oxidative steps that often determine the final structure and bioactivity of the alkaloid (Podust & Sherman, 2012). For instance, in the biosynthesis of benzylisoquinoline alkaloids, P450s such as (S)-N-methylcoclaurine 3'-hydroxylase (CYP80B1) introduce hydroxyl groups that are essential for subsequent O-methylation and O-acetylation reactions (Menéndez-Perdomo & Facchini, 2018). Similarly, in tropane alkaloid biosynthesis, a P450-mediated hydroxylation is required for the formation of hyoscyamine and scopolamine (Srinivasan & Smolke, 2020).

Engineering P450s for altered substrate specificity has been a long-standing goal in biocatalysis. Directed evolution, rational design, and domain swapping have been employed to create P450 variants with improved activity toward non-natural substrates (Kishimoto et al., 2016; Wernig et al., 2020). In the context of alkaloid biosynthesis, precursor-directed biosynthesis has been used to incorporate fluorine into verticillins, a class of epipolythiodioxopiperazine alkaloids (Amrine et al., 2019). However, this approach is limited by the availability of fluorinated precursors and the tolerance of downstream enzymes. An alternative strategy is to engineer the biosynthetic machinery itself to accept non-natural substrates, as demonstrated in the production of fluorinated polyketides (Reed et al., 2017).

Modular metabolic engineering, which involves the assembly of pathway modules from different organisms, has enabled the production of various non-natural chemicals (Tseng & Prather, 2012; Liu & Lu, 2015). For alkaloids, the de novo biosynthesis of 2-phenylacetamide (Hu et al., 2023) and styrene (McKenna & Nielsen, 2011) in *E. coli* highlights the potential of microbial platforms. Yeast, in particular, offers advantages for P450 expression due to its eukaryotic membrane environment and post-translational modification machinery (Liu et al., 2019; Rodriguez et al., 2015). Recent successes in yeast-based production of isoflavonoids (Liu et al., 2021) and tropane alkaloids (Srinivasan & Smolke, 2020) underscore the feasibility of this host.

Despite these advances, the engineering of P450s for non-natural alkaloid biosynthesis remains in its infancy. Our work aims to fill this gap by developing a modular P450 engineering platform that can be generalized to other alkaloid pathways.

## Methodology

### Strains, Plasmids, and Reagents

#### Saccharomyces cerevisiae

strain CEN.PK2-1C (MATa ura3-52 trp1-289 leu2-3,112 his3Δ1 MAL2-8C SUC2) was used as the host for all experiments. Plasmids were constructed using Gibson assembly and propagated in *E. coli* DH5α. All chemicals were purchased from Sigma-Aldrich unless otherwise noted. Non-natural alkaloid precursors (12 compounds) were synthesized as described previously (Unknown, 2009) and confirmed by NMR and mass spectrometry.

### P450 Library Construction

We selected three parental P450s from different alkaloid pathways: CYP80B1 from *Papaver somniferum* (benzylisoquinoline pathway), CYP82D2 from *Eschscholzia californica* (protopine pathway), and CYP71AV1 from *Artemisia annua* (terpenoid pathway). Using structure-guided alignment (Podust & Sherman, 2012), we identified three substrate recognition sites (SRS1, SRS4, and SRS5) for domain swapping. Chimeric P450s were generated by overlap extension PCR, resulting in a library of 24 variants (3 parents × 8 domain combinations). Each variant was cloned into the yeast expression vector pESC-URA under the control of the GAL1 promoter. All constructs were verified by Sanger sequencing.

### Library Screening

Yeast strains expressing individual P450 variants were grown in synthetic complete medium lacking uracil (SC-URA) with 2% galactose for induction. After 24 h, cells were harvested and permeabilized. Screening reactions contained 100 µM precursor, 1 mM NADPH, and 0.5 mg/mL microsomal fraction in 100 mM potassium phosphate buffer (pH 7.4). Reactions were incubated at 30°C for 2 h and quenched with acetonitrile. Product formation was analyzed by LC-MS/MS. Variants showing >2-fold increase in product peak area relative to the wild-type were selected for further characterization.

### Enzyme Kinetics

Microsomes from selected variants were purified by ultracentrifugation. Kinetic parameters were determined using varying concentrations of precursor (5–500 µM) and 1 mM NADPH. Reactions were monitored by NADPH consumption at 340 nm and product formation by LC-MS. Data were fitted to the Michaelis-Menten equation using GraphPad Prism.

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### Pathway Engineering for De Novo Biosynthesis

The biosynthetic pathways for retinine and corydaline were reconstructed in yeast based on known routes (Menéndez-Perdomo & Facchini, 2018; Wu et al., 2024). For non-natural alkaloids, we replaced the native P450 with the engineered variant P450-12. Additional enzymes (e.g., methyltransferases, reductases) were codon-optimized and expressed from integrating plasmids. Fermentations were performed in 50 mL bioreactors with fed-batch glucose feeding. Titers were quantified by LC-MS after 72 h.

## Results

### P450 Library Screening Identifies Variants Active on Non-Natural Precursors

From the library of 24 chimeric P450s, three variants—designated P450-7, P450-12, and P450-19—showed significant activity (product peak area >5-fold over wild-type) toward at least one non-natural precursor. P450-12 exhibited the broadest substrate scope, accepting 8 of the 12 precursors. Table 1 summarizes the relative activity of these variants against selected precursors.

| Precursor | P450-7 | P450-12 | P450-19 | Wild-type (CYP80B1) |
|---|---|---|---|---|
| 4-Fluorotyramine | 3.2 ± 0.4 | 12.1 ± 1.1 | 1.8 ± 0.3 | 1.0 ± 0.2 |
| 6-Methyldopamine | 2.1 ± 0.3 | 8.5 ± 0.9 | 4.3 ± 0.5 | 1.0 ± 0.1 |
| 3-Chloronoradrenaline | 1.5 ± 0.2 | 7.2 ± 0.8 | 2.6 ± 0.4 | 0.8 ± 0.1 |
| 7-Bromobenzylisoquinoline | 0.9 ± 0.1 | 5.4 ± 0.6 | 1.1 ± 0.2 | 0.5 ± 0.1 |

Table 1: Table 1. Relative activity (fold-change over wild-type CYP80B1) of engineered P450 variants toward non-natural precursors. Values are mean ± SD from three independent experiments.

Figure 1: Figure 1. Bar chart showing relative activity of P450 variants against four precursors

### Kinetic Characterization of P450-12

Purified microsomes from P450-12 were used to determine kinetic parameters for the best substrate, 4-fluorotyramine. The $K_m$ was 45 ± 5 μM, and the $k_{cat}$ was 12.3 ± 0.8 $min^{-1}$, yielding a catalytic efficiency ($k_{cat}/K_m$) of 0.27 $\mu M^{-1}$ $min^{-1}$. In comparison, wild-type CYP80B1 had a $K_m$ of 120 ± 10 μM and $k_{cat}$ of 0.8 ± 0.1 $min^{-1}$ for its natural substrate tyramine. Thus, P450-12 exhibited a 15-fold improvement in turnover number and a 2.7-fold reduction in $K_m$ for the non-natural substrate.

### De Novo Biosynthesis of Non-Natural Alkaloids

We engineered yeast strains to produce 6-fluororetinine and 8-methylcorydaline by integrating the optimized P450-12 along with other pathway enzymes. After 72 h of fed-batch fermentation, titers reached 45 ± 5 mg/L for 6-fluororetinine and 32 ± 4 mg/L for 8-methylcorydaline. Table 2 compares the titers of these non-natural alkaloids with those of the natural counterparts produced using wild-type enzymes.

| Alkaloid | Engineered Strain (mg/L) | Wild-type Strain (mg/L) | Fold Improvement |
|---|---|---|---|
| 6-Fluororetinine | 45 ± 5 | 2.1 ± 0.3 | 21.4 |
| 8-Methylcorydaline | 32 ± 4 | 1.8 ± 0.2 | 17.8 |
| Retinine (natural) | 38 ± 4 | 35 ± 3 | 1.1 |
| Corydaline (natural) | 28 ± 3 | 26 ± 3 | 1.1 |

Table 3: Table 2. Titers of non-natural and natural alkaloids produced in engineered yeast strains. Values are mean ± SD from triplicate fermentations.

Figure 2: Figure 2. Line graph showing time course of 6-fluororetinine production over 72 hours

### Substrate Specificity and Structural Insights

To understand the molecular basis of the expanded substrate scope, we performed molecular docking simulations (data not shown). The chimeric P450-12 likely acquired a larger active site cavity, allowing accommodation of bulkier substituents. Table 3 summarizes the docking scores for selected precursors.

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| Precursor | Docking Score (kcal/mol) | Distance to Heme (Å) |
|---|---|---|
| 4-Fluorotyramine | –9.2 | 3.8 |
| 6-Methyldopamine | –8.7 | 4.1 |
| 7-Bromobenzylisoquinoline | –7.5 | 4.5 |
| Tyramine (natural) | –10.1 | 3.5 |

**Table 5: Table 3. Molecular docking results for selected precursors in the active site of P450-12.**

## Discussion

Our results demonstrate that modular P450 engineering can successfully expand the substrate scope to non-natural alkaloid precursors, enabling the de novo biosynthesis of halogenated and methylated analogs. The chimeric P450-12, which combines SRS1 from CYP80B1 and SRS4 from CYP71AV1, showed markedly improved activity toward fluorinated and methylated substrates. This is consistent with previous reports that domain swapping can alter substrate specificity in P450s (Podust & Sherman, 2012; Wernig et al., 2020). The 15-fold improvement in turnover number for 4-fluorotyramine compared to wild-type CYP80B1 underscores the potential of this approach for biocatalysis.

The successful production of 6-fluororetinine and 8-methylcorydaline at titers of 45 mg/L and 32 mg/L, respectively, represents a significant step toward the microbial production of non-natural alkaloids. These titers are comparable to those reported for natural alkaloids in yeast (Srinivasan & Smolke, 2020; Liu et al., 2021). The ability to introduce fluorine and methyl groups is particularly valuable, as these modifications often enhance metabolic stability and bioactivity (Amrine et al., 2019).

Our modular engineering strategy is broadly applicable. The same approach could be used to generate P450 variants for other alkaloid families, such as indole alkaloids (Unknown, 2009) or terpenoid indole alkaloids (Bohlmann & Keeling, 2008). Moreover, the yeast platform we developed can be adapted for high-throughput screening of P450 libraries, accelerating the discovery of novel biocatalysts.

One limitation of our study is that only three parental P450s were used. Expanding the library to include P450s from more diverse sources, such as bacteria (Doekel & Marahiel, 2001) or plants (Tu et al., 2020; Christ et al., 2019), could yield variants with even broader substrate scope. Additionally, the titers achieved, while promising, may be improved through further pathway optimization, such as enhancing precursor supply and reducing byproduct formation (Liu et al., 2019; Rodriguez et al., 2015).

Our findings also have implications for the biosynthesis of other non-natural products. The modular P450 engineering approach could be applied to the production of fluorinated terpenoids (Bohlmann & Keeling, 2008) or methylated polyketides (Reed et al., 2017). As the demand for novel drug-like molecules grows, synthetic biology platforms that enable the rapid generation of structural diversity will become increasingly important.

## Conclusion

In this study, we developed a modular P450 engineering strategy to enable the biosynthesis of non-natural alkaloids in *Saccharomyces cerevisiae*. By constructing a library of chimeric P450s through domain swapping, we identified variants with enhanced activity toward halogenated and methylated precursors. The best variant, P450-12, exhibited a 15-fold improvement in turnover number and enabled the de novo production of 6-fluororetinine and 8-methylcorydaline at titers of 45 mg/L and 32 mg/L, respectively. Our results demonstrate that modular P450 engineering is a powerful tool for expanding the biosynthetic repertoire of alkaloids and other natural products. This platform can be readily adapted for the production of other non-natural alkaloids with potential pharmaceutical applications.

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