Microbial Factories for the Production of Benzylisoquinoline Alkaloids

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Benzylisoquinoline alkaloids (BIAs) are a family of ~2500 alkaloids with both potential and realized pharmaceutical value, including most notably the opiates such as codeine and morphine. Only a few BIAs accumulate readily in plants, which limits the pharmaceutical potential of the family. Shifting BIA production to microbial sources could provide a scalable and flexible source of these compounds in the future. This review details the current status of microbial BIA synthesis and derivatization, including rapid developments in the past 6 months culminating in the synthesis of opioids from glucose in a microbial host.

Microbial Synthesis of Pharmaceuticals To Enhance Drug Discovery

Plant secondary metabolites are a valuable source of natural products with pharmaceutical properties ¹,². Until recently pharmaceutical companies had phased out screening of natural products as potential drug leads in favor of chemically synthesized libraries, citing ‘poor yields of chemical synthesis’ and ‘impracticality of scale-up’ ³,⁴. While these challenges remain, there has been a resurgence of natural products in the drug discovery process owing to their potent biological activity and untapped potential, and recent improvements in screening technology ⁴,⁵.

Biosynthesis of plant metabolites in microbes provides an opportunity to advance the drug discovery process. Microbial production circumvents the need for the cultivation of source plants ⁶. Not all products accumulate to high levels in plants, and hence microbes provide a way to scale up production of these interesting compounds to relieve supply limitations ⁷. The successful industrial production of the antimalarial artemisinic acid in yeast highlights the capability of microbes to act as natural product factories ⁸. Finally, microbial production facilitates the possibility to design new compounds with novel activity or improve the clinical profile of existing drugs through combinatorial chemistry ⁹.

Pharmaceutical Properties of Benzylisoquinoline Alkaloids

Benzylisoquinoline alkaloids (BIAs) are a class of molecules that would benefit from microbial synthesis. With over 2500 family members, BIAs exhibit diverse pharmaceutical properties, with a history of human use dating back thousands of years ¹⁰. In addition to their prominent role in traditional medicine, BIAs have a wide variety of pharmacological applications, acting as analgesics, antitussives, antimicrobials, and antispasmodics, and several members are on the World Health Organization list of essential medicines (Table 1). Recently, preliminary studies have uncovered new potential in treating cancer, malaria, HIV, and psychosis (Table 1). Despite their clinical applications, it is argued that BIAs (among other alkaloids) are not proportionately represented in modern medicine and drug development, in large part owing to difficulties in

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To overcome this barrier, several groups have been working to synthesize and derivatize BIAs in the microbial hosts Escherichia coli and Saccharomyces cerevisiae.

### BIA Diversity Is Derived from a Single Scaffold

BIA synthesis in plants begins with the condensation of dopamine and 4-hydroxyphenyacetalddehyde (4-HPAA) to form (S)-norcoclaurine (Figure 1A). (S)-norcoclaurine is the scaffold from which over 2500 other BIAs can be produced (examples of the structural diversity of BIAs are provided in Figure 1B) [12]. This means that a microbial platform strain capable of producing the BIA scaffold from simple carbon sources could then be engineered to produce any BIA of interest, provided the necessary enzymes have been elucidated.

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**Table 1. Diversity of BIAs in Drug Development**

<table>
<thead>
<tr>
<th>BIA Subfamily</th>
<th>Compound</th>
<th>Pharmaceutical Applications</th>
<th>Clinical Status</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzylisoquinoline</td>
<td>Norcoclaurine</td>
<td>Cardiac Stimulant</td>
<td>Clinical trials (Phase 1)</td>
<td>[68–70]</td>
</tr>
<tr>
<td></td>
<td>Drotaverine</td>
<td>Antispasmodic</td>
<td>Approved</td>
<td>[68,71]</td>
</tr>
<tr>
<td></td>
<td>Papaverine</td>
<td>Vasodilator</td>
<td>Approved</td>
<td>[68,71,72]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Smooth muscle relaxant</td>
<td>Approved</td>
<td></td>
</tr>
<tr>
<td>Bisbenzylisoquinoline</td>
<td>Atracurium&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Neurmuscular blocker</td>
<td>Approved</td>
<td>[71,73]</td>
</tr>
<tr>
<td></td>
<td>Mivacurium&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Neurmuscular blocker</td>
<td>Approved</td>
<td>[81,74]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Muscle relaxant</td>
<td>Approved</td>
<td></td>
</tr>
<tr>
<td>Phthalidisoquinoline</td>
<td>Noscapine</td>
<td>Antitussive</td>
<td>Approved</td>
<td>[72,75]; Drugs.com (<a href="http://www.drugs.com/international/noscapine.html">www.drugs.com/international/noscapine.html</a>)</td>
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<tr>
<td></td>
<td></td>
<td>Potential Anticancer</td>
<td>Clinical trials (Phase 2)</td>
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<td></td>
<td></td>
<td>Antimalarial</td>
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<td></td>
</tr>
<tr>
<td>Aporphine</td>
<td>Glaucine</td>
<td>Antitussive</td>
<td>Approved</td>
<td>[68,76]; Drugs.com (<a href="http://www.drugs.com/international/glaucine.html">www.drugs.com/international/glaucine.html</a>)</td>
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<td></td>
<td></td>
<td>Anticancer</td>
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<td></td>
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<tr>
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<td>Berberine</td>
<td>HIV treatment</td>
<td>N/A</td>
<td>[68,71,72,77–79]</td>
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<tr>
<td></td>
<td></td>
<td>Antibacterial</td>
<td>N/A</td>
<td></td>
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<td>Experimental</td>
<td></td>
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<td></td>
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<td>Antifungal</td>
<td>Experimental</td>
<td></td>
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<td></td>
<td></td>
<td>Antibacterial</td>
<td>Experimental</td>
<td></td>
</tr>
<tr>
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<td></td>
<td>Antidiarrheal</td>
<td>Experimental</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Type II diabetes</td>
<td>Clinical trials (Phase 3)</td>
<td></td>
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<tr>
<td></td>
<td>Stepholidine</td>
<td>Psychosis</td>
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<td>[80]</td>
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<tr>
<td>Benzophenanthridine</td>
<td>Sanguinarine</td>
<td>Antimicrobial</td>
<td>Not approved</td>
<td>[68,81]</td>
</tr>
<tr>
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<td>Codeine</td>
<td>Analgesic</td>
<td>Approved</td>
<td>[68,71,72]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antitussive</td>
<td>Approved</td>
<td></td>
</tr>
<tr>
<td>Morphinan semisynthetic derivatives</td>
<td>Oxycodone&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Analgesic</td>
<td>Approved</td>
<td>[50,68,71]</td>
</tr>
<tr>
<td></td>
<td>Naloxone&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Opioid antagonist</td>
<td>Approved</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Naltrexone&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Opioid antagonist</td>
<td>Approved</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Synthetic or semi-synthetic BIAs.
Figure 1. Microbial Synthesis of BIAs and BIA diversity. (A) 4-HPP is the microbial precursor to the substrates of norcoclaurine/norlaudanosine condensation. Dopamine and 4-HPAA condense to form norcoclaurine (black), while dopamine and 3,4-dHPAA condense to form norlaudanosine (red). The extra hydroxyl group on 3,4-dHPAA and norlaudanosine is indicated in red, and reactions that accept norlaudanosine are indicated with red arrows. Metabolite abbreviations: 4-HPP, 4-hydroxyphenylpyruvate; 4-HPAA, 4-hydroxyphenylacetaldehyde; 3,4-dHPAA, 3,4-dihydroxyphenylacetaldehyde. Enzyme abbreviations: AADC, amino acid decarboxylase; AAT, amino acid transferase; CNMT, covaline N-methyltransferase; NCS, norcoclaurine synthase; NMCH, N-methyccoclaurine 3′-O-hydroxylase; 4′OMT, 3′-hydroxy-N-methylcoclaurine 4′-O-methyltransferase; 6OMT, norcoclaurine 6-O-methyltransferase; TYR, any enzyme which hydroxylates tyrosine. (B) Diversity of BIA scaffolds. Featured BIAs are examples of indicated scaffold types. The number of arrows is not indicative of pathway length. Dashed lines indicate unknown pathways; blue lines indicate one or more cytochrome P450-catalyzed reactions.

Ten enzyme families add functional groups and catalyze the rearrangement of BIA scaffolds [10,13]. Many of these enzymes are cytochrome P450s that require endomembranes for optimal activity (Figure 1B, blue arrows). For this reason, S. cerevisiae has been used extensively for heterologous BIA pathway development, although ample successes have been achieved in E. coli. Enzymes in BIA synthesis tend to have broad substrate ranges, allowing the same reactions to be performed even as BIAs diverge in structure (see [14–18] for examples). Although BIA synthesis pathways are commonly drawn in straight lines, the substrate acceptance profiles of enzymes in a single pathway can overlap, meaning that these pathways are often more of a web resulting in a multitude of products (an example is the morphine pathway, shown in Figure 2). Metabolic engineering strategies will be necessary to overcome promiscuity for the reconstitution of BIA synthesis pathways in heterologous hosts (Box 1).

Current Status of Microbial Aromatic Amino Acid (AA) Production

The (S)-norcoclaurine precursors 4-HPAA and dopamine are derived from the aromatic AA pathway, and hence aromatic AA overproduction is a key goal for the development of microbial sources of BIAs. Entry of carbon into the aromatic AA pathway begins with the condensation of the glycolysis intermediate phosphoenolpyruvate and the pentose phosphate pathway intermediate erythrose-4-phosphate. This committed step is transcriptionally and allosterically regulated, which is a common theme throughout the aromatic AA pathway, especially for enzymes at key metabolic branch-points [19]. Strategies for improving flux towards tyrosine in E. coli and S. cerevisiae are similar, but the highest published yields of aromatic AAs or
Figure 2. Synthesis of Morphinan Alkaloids and Derivatives in Saccharomyces cerevisiae. Epimerization of (S)-reticuline to (R)-reticuline and spontaneous rearrangement of salutaridinol-7-O-acetate to thebaine mark the starting points for the synthesis of pro-morphinan and morphinan alkaloids, respectively. Colored lines indicate overlapping biosynthetic pathways leading to opiates and opiate derivatives with pharmaceutical properties. Pink, synthesis of morphine from thebaine via codeine; purple, synthesis of morphine from thebaine via oripavine; yellow, synthesis of hydromorphone via morphine; green, synthesis of hydromorphone via oripavine; blue, synthesis of hydrocodone and oxycodone from thebaine. Promiscuous enzymes are indicated in colored boxes: thebaine 6-O-demethylase (T6ODM, blue), codeine demethylase (CODM, red), Pseudomonas putida morphine reductase (morB, purple), codeine reductase (COR, and P. putida morphine dehydrogenase (morA), which catalyze most of the same reactions, are in green. Spontaneous (Sp) rearrangements, which favor synthesis of additional substrates for promiscuous enzymes, are indicated in yellow. Other abbreviations: CPR, cytochrome P450 reductase; REP, reticuline epimerase; SAS, salutaridine synthase (CYP719B1); SAR, salutaridine reductase; SAT, salutaridinol 7-O-acetyltransferase.
Box 1. Strategies for Reducing Side-Products in BIA Synthesis Pathways

At least 37% of E. coli enzymes can accept more than one substrate [82], and this percentage increases for enzymes involved with secondary metabolism [83]. The broad substrate range of enzyme families in BIA synthesis has been demonstrated in vitro (examples include [14–18]). Consequently, reconstitution of BIA synthesis pathways has resulted in the generation of side-products in microbial hosts (Figure A) for both the morphine [53] and sanguinarine pathways [61].

Promiscuity presents both a challenge and an opportunity: As a positive, promiscuous enzymes can be used to catalyze reactions for which a dedicated enzyme has not been identified (reviewed in [84]). For example, a promiscuous N-methyltransferase was used to methylate BIA structures other than those for which it was characterized [49]. While not always ideal, initial successes can be a starting point for mutagenesis or directed evolution to promote the desired activity [85,86]. However, directed evolution begins with an effective screening strategy. A colorimetric sensor has been used to improve the synthesis of the BIA precursor L-DOPA [38], but an efficient screen remains to be developed for downstream BIA derivatization.

The effects of promiscuity can be mediated by improving flux through the appropriate pathway, or through spatial or temporal sequestration of enzymes away from pathway intermediates. General flux improvement strategies (reviewed in [87]), such as modulation of gene copy number [46,53] and promoter strength (Figure B) [48,58], have improved yield in BIA synthesis pathways. Enzyme scaffolding could also push flux through the intended pathway (Figure C). Sequestration of promiscuous enzymes into other organelles [53] or separate engineered microbes [37] has improved synthesis of BIs (Figure E). Alternatively, temporal control at the level of transcriptional regulation could allow buildup of the desired intermediate before the expression of a promiscuous enzyme (Figure E).

Re-engineering the pathway itself can also be used to avoid promiscuous side-reactions. For example, non-productive side-products could be brought back into the main pathway through the co-expression of other promiscuous enzymes (Figure F). Alternatively, the substrate acceptance profiles of each enzyme could be matched to avoid the generation of side-products, recently demonstrated by the heterologous synthesis of >90% unique carotenoids using only promiscuous enzymes [88]. Finally, the use of enzymes to protect functional groups from unwanted side-activities has recently been described in opium poppy [82]. This raises the possibility of re-engineering pathways to include blocking steps to avoid promiscuous enzymes, followed by later removal of the group (Figure F), a method commonly used in synthetic organic chemistry.

Figure I. Strategies for the Reduction of Side-Products in Pathways with Promiscuous Enzymes. (A) An initial pathway that results in the accumulation of side-products. (B) DNA copy number, ribosome binding site (RBS) strength, and promoter strength can be adjusted to improve flux by modulating enzyme expression (thinner pipe, less enzyme; fatter pipe, more enzyme; small red dot, less side-product). (C) Enzyme scaffolding (black line and shorter pipe) can prevent access of intermediates to promiscuous enzymes. (D) Enzymes can be physically separated into subcellular compartments or between microbial strains (black boxes). (E) Expression of a promiscuous enzyme can be delayed (− ind) until an inducer is added or growth conditions result in transcription (+ ind). (F) Alternative enzymes can be expressed to adjust the pathway such that side-products are not produced.
aromatic AA-derived compounds are currently an order of magnitude higher in *E. coli* than in yeast, both in shake-flask (∼300 mg/l vs 2 g/l) [20–22] and fermentation (2 g/l vs 55 g/l) conditions [23,24]. Notably, the shift from laboratory shake-flask to industrial fermentation can improve total titers, although not necessarily yield, by one to two orders of magnitude. A striking example of this is the development of fermentation conditions for a yeast strain to synthesize artemisinic acid, in which titers of the intermediate amorpha-4,11-diene increased from 160 mg/l to 40 g/l [25]. Although BIA titers reported throughout this review are low, much of the work was done in shake flasks, suggesting that optimized fermentation conditions combined with the latest developments in strain engineering will easily improve upon these titers.

Aldehyde Scavenging Limits the Available 4-HPAA Pool

Because the aldehyde 4-HPAA is a substrate of norcoclaurine synthesis, its *in vivo* stability is necessary for BIA overproduction in microbes. However, aldehydes are rapidly scavenged to limit cellular toxicity [26]. Aldehydes can be reduced or oxidized depending on the redox status of the cell [27]. No less than six aldehyde dehydrogenases (ALDs) and 16 alcohol dehydrogenases (ADHs) can participate in aldehyde reduction/oxidation in yeast, with significant redundancy [27]. *E. coli* also harbors multiple ALDs and ADHs, whose knockout has improved heterologous production of aldehydes [28,29]. Preliminary data suggests that ALD and ADH knockouts may improve *de novo* BIA production in yeast [30].

Multiple Strategies for Dopamine Synthesis in Microbial Hosts

The formation of dopamine from tyrosine requires one hydroxylation and one decarboxylation event (Figure 1A). Depending on enzyme specificity, these reactions could occur in either order; when decarboxylation occurs first the intermediate is tyramine, while if hydroxylation occurs first the intermediate is L-DOPA. Thus far, the decarboxylation-first pathway has been avoided through the use of a decarboxylase that has a strong preference for L-DOPA [31]. This is historically because production of L-DOPA, as opposed to tyramine, made downstream pathway engineering easier in *E. coli* [31]. L-DOPA has remained the intermediate of choice as *de novo* BIA synthesis has been introduced to yeast.

Many options for heterologous L-DOPA synthesis exist, with no one enzyme standing out as being clearly superior to other enzymes. Currently, enzyme selection for L-DOPA synthesis requires choosing between undesired side-activities and the requirement for a cofactor. There are two types of side-activities relating to L-DOPA synthesis: broad substrate range and L-DOPA oxidation to dopaquinone (diphenolase activity). Tyrosinases (TYR) and hydroxyphenylacetic acid hydroxylases (HPAH) have broad substrate ranges [32,33], while TYRs and the cytochrome P450 hydroxylase CYP76AD1 have diphenolase activity on L-DOPA [34,35]. While catalyzing two types of side activity, TYR has the lowest cofactor requirement (some require only inorganic copper). By contrast, tyrosine hydroxylase (TH) has the lowest amount of side-activity but the highest cofactor requirements because it uses the cofactor tetrahydrobioprotein (BH$_4$). BH$_4$ is not native to *E. coli* and *S. cerevisiae*, and hence the functional expression of TH also requires the heterologous expression of a BH$_4$ synthesis and regeneration pathway. THs have an additional disadvantage in that they are heavily regulated by allosteric inhibition and post-translational modification reflecting their role as the rate-limiting step of catecholamine synthesis in neurons [36].

The disadvantages of various enzyme families have been addressed during the introduction of L-DOPA synthesis in both *E. coli* and *S. cerevisiae*. A TYR with an unusually low level of diphenolase activity (RsTYR) was expressed in *E. coli* for heterologous BIA synthesis, resulting in the production of 2.5 g/l dopamine [37]. However, RsTYR still possessed some diphenolase activity and its broad substrate range allowed it to oxidize downstream BIAs. This has since been addressed by using multiple strains of *E. coli* to sequester RsTYR from downstream BIA
synthesis enzymes [37]. However, this multistrain system is not without drawbacks (Box 2). While HPAH also has a broad substrate range, it does not oxidize its products, and hence HPAH may be a better option than RsTYR for L-DOPA synthesis in E. coli.

HPAH has not been demonstrated to be functional in yeast, and TYR activity in yeast is low [38], pointing towards TH and CYP76AD1 as better options for L-DOPA production in S. cerevisiae. The side-activity of CYP76AD1 was reduced by subjecting it to mutagenesis followed by screening with a color-based biosensor that can detect and distinguish between L-DOPA and dopaquinone synthesis [38]. Alternatively, the BH₄ synthesis pathway has now been introduced into S. cerevisiae [39,40], permitting functional TH expression for BIA synthesis [40]. The use of either enzyme to produce dopamine, regardless of strain engineering strategy, currently results in yields of ~10–25 mg/l. These titers are lower than the 2.5 g/l dopamine produced in E. coli, but are not uncommon for heterologous products derived from aromatic AAAs in yeast [22,41], which points to the necessity to improve the synthesis of precursors to achieve higher dopamine levels. Recent titers of 2 and 3 g/l of aromatic AA pathway derivatives in yeast indicate that engineering strategies are being developed that should further improve BIA yields [24,42].

**BIA Scaffolds Can Be Synthesized De Novo from Simple Sugars**

Pictet–Spengler condensation of an amine and aldehyde is a reaction mechanism common to the committed step of several alkaloid families [43]. Spontaneous condensation generates racemic (R,S) mixtures, whereas enzymatic condensation is enantio-specific. The committed step of BIA synthesis is enzymatically catalyzed by norcoclaurine synthase (NCS), which condenses dopamine and 4-HPAA to generate (S)-norcoclaurine. Because BIAs are derived from (S)-norcoclaurine, the synthesis of (R)-norcoclaurine is unproductive, and hence enzymatic condensation is preferable to spontaneous condensation for microbial production of BIA scaffolds. NCS has a broad substrate range for aldehydes. In addition to 4-HPAA, NCS can accept the double-hydroxylated 3,4-dHPAA, which when condensed with dopamine generates (S)-norlaudanosoline (Figure 1A). Norlaudanosoline has been used extensively for BIA derivatization in E. coli because the extra hydroxyl group on 3,4-dHPAA negates the need for later cytochrome P450-catalyzed hydroxylation of the BIA scaffold (Figure 1A) [44]. Both norcoclaurine and norlaudanosoline are unstable end-products because they are subject to enzymatic

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**Box 2. Fermentation Conditions for BIA Synthesis**

The pH of fermentation affects BIA yields, both synthesis and derivatization, in E. coli and in yeast. E. coli fermentations are usually performed at pH 7, which is sufficiently alkaline for the non-productive spontaneous oxidation of L-DOPA, dopamine, and norlaudanosine [31]. pH 6 was found to be the best compromise between E. coli growth and de novo BIA synthesis [46]. However, the continued disappearance of norlaudanosoline in supernatant indicates that these conditions are still not optimal [37].

The pH of yeast cultures (3–6) is lower than E. coli cultures, which reduces spontaneous oxidation of norlaudanosoline and precursors in supernatant. However, BIA (and other alkaloid) [39] derivatization from supplemented precursors is more efficient at higher pHs. As pH is increased from 3 to 8, a greater fraction of supplemented BIAs are associated with cell extracts, and conversion to downstream products is higher [89]. While supplemented precursors represent an intermediate step to a final production strain, endogenously produced dopamine, norcoclaurine, and reticuline are primarily found in yeast supernatant [38], and typically do not re-enter cells efficiently once outside [40]. Knockout of transporters to prevent secretion is an alternative method for controlling ratios of BIA fractionation [90]. However, secretion of end-products would make downstream industrial processing easier. Importantly, the fractionation of BIAs as well as the rate of flux through BIA synthesis pathways must be balanced for the greatest pathway efficiency.

Groups working with E. coli and yeast have explored other fermentation conditions for de novo synthesis and derivatization of BIAs in vivo. Growth at lower temperatures improves BIA conversion in both species [46,48]. Alternative carbon sources can improve the production of precursors [31] and derivatization of downstream BIAs [48]. Conditions such as pH, temperature, and carbon source can be adjusted throughout fermentative production. An initial accumulation of biomass before heterologous compound production, such as has been done for production of 1,3-propanediol in E. coli [91], has also improved both the synthesis of aromatic AA pathway derivatives [42] as well as the derivatization of BIAs [48,56,61].
oxidation as well as spontaneous oxidation at alkaline pHs. Therefore, the key branch-point intermediate reticuline (Figure 1B), derived from norcoclaunine/norlaudanosoline (Figure 1A), is frequently used as a readout for de novo synthesis of norcoclaunine/norlaudanosoline.

Norlaudanosoline Synthesis in E. coli
3,4-dHPAA can be generated from dopamine via monoamine oxidase (MAO), making dopamine the source of both amine and aldehyde for norlaudanosoline synthesis (Figure 1A). As a first proof of concept for the synthesis of BIA in a microbial host, reticuline was produced from supplemented dopamine in E. coli [45]. Initially, 1.3% of supplemented dopamine was converted to reticuline, which was improved in later studies after optimizing both fermentation conditions and gene copy-number (Box 2, Table 2) [46]. The further introduction of endogenous dopamine synthesis to E. coli enabled fermentation of reticuline from glycerol, with an initial dopamine-to-reticuline conversion of 4% (Table 2) [31].

Enzymatic oxidation of norlaudanosoline was identified as a side-activity of TYR that reduced conversion of dopamine to reticuline. To prevent TYR activity on norlaudanosoline, norlaudanosoline synthesis was divided between two strains of E. coli (glycerol-to-dopamine and dopamine-to-norlaudanosoline), which were cultured sequentially (Box 1). Preventing diphenolase activity on norlaudanosoline improved its accumulation 300-fold, resulting in 16% conversion from dopamine. However, the addition of a third strain to convert norlaudanosoline to reticuline did not improve total dopamine-to-reticuline yields compared to previous results (Table 2). It is possible that this discrepancy is due to the extended time that norlaudanosoline was exposed to culture supernatant, which promotes spontaneous oxidation (Box 2). Norlaudanosoline oxidation in supernatant may be avoided in the future by re-engineering the multi-strain system such that dopamine produced by the first strain is converted directly to reticuline by a second strain expressing MAO as well as the reticuline synthesis pathway.

Table 2. Synthesis of Reticuline in Microbial Hosts

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dopamine</th>
<th>Norlaudanosoline</th>
<th>Reticuline</th>
<th>% Yield</th>
<th>Comments</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>Supplement, 5 mM</td>
<td>–</td>
<td>33 µM</td>
<td>1.3</td>
<td>First synthesis of reticuline in a microbial host</td>
<td>[45]</td>
</tr>
<tr>
<td>E. coli</td>
<td>Supplement, 3 mM</td>
<td>–</td>
<td>165 µM</td>
<td>11</td>
<td>Improved fermentation conditions</td>
<td>[46]</td>
</tr>
<tr>
<td>E. coli</td>
<td>De novo, 7 mM*</td>
<td>–</td>
<td>140 µM</td>
<td>4</td>
<td>First de novo BIA synthesis. Fed-batch</td>
<td>[31]</td>
</tr>
<tr>
<td>E. coli</td>
<td>De novo, 14 mM*</td>
<td>–</td>
<td>145 µM</td>
<td>2</td>
<td>Three-step fermentation. Fed-batch</td>
<td>[37]</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>–</td>
<td>Supplement, 4 mM</td>
<td>455 µM</td>
<td>10</td>
<td>First derivatization of norlaudanosoline in S. cerevisiae</td>
<td>[58]</td>
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<tr>
<td>S. cerevisiae</td>
<td>–</td>
<td>Supplement, 0.01 mM</td>
<td>2 µM</td>
<td>20</td>
<td>Improved yield</td>
<td>[61]</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>De novo, 0.155 mM*</td>
<td>–</td>
<td>0.2 µM</td>
<td>0.13</td>
<td>First de novo BIA synthesis in S. cerevisiae. Shake-flask</td>
<td>[38]</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>De novo, 0.065 mM*</td>
<td>–</td>
<td>0.2 µM</td>
<td>0.31</td>
<td>Improved yield. Shake-flask</td>
<td>[52]</td>
</tr>
</tbody>
</table>

* Molar yield.
* Dopamine quantified in supernatant of strains expressing no downstream enzymes.
* Reticuline concentration estimated based on closest available standard.
**Norcoclaurine Synthesis in S. cerevisiae**

This year, two groups have achieved BIA synthesis from simple carbon sources in yeast. Neither group used MAO to generate 3,4-dHPAA from dopamine. Instead, endogenous cytosolic 4-HPAA was the source of the aldehyde. Both groups reported comparable reticuline yields several orders of magnitude lower than the most recent yields published for E. coli (Table 2). In particular, the drop in titers from dopamine (~10–25 mg/l) to norcoclaurine (~80–100 μg/l) indicates that norcoclaurine synthase is a key bottleneck in the de novo synthesis of BIAs in yeast [38].

Supplementation of dopamine for spontaneous condensation to norcoclaurine in yeast was much less efficient than in E. coli (0.0025% vs 16%) [40]. Endogenously produced dopamine was also converted to reticuline at lower levels in yeast than in E. coli (0.3% vs 4%) (Table 2). Considering that endogenously produced dopamine is found in the supernatant [38], low norcoclaurine yields could in part be due to dopamine secretion occurring more readily than norcoclaurine synthesis can occur. The KM for dopamine for the NCS of Thalictrum flavum is 25 mM [47], which is approximately double the concentration of dopamine produced in E. coli, but 170-fold of that currently produced in yeast. It is possible that dopamine values in yeast are currently too low for efficient synthesis of norcoclaurine. Local dopamine concentrations could be improved further via strain engineering to improve titers, reduce efflux, or sequester dopamine in subcellular compartments.

Currently, as a percentage of dopamine, reticuline yields from groups working in S. cerevisiae are similar to the first numbers published in E. coli (Table 2). Increases in E. coli yields were incremental, and required optimization of strain design and fermentation conditions [31,37,45,46]. Fermentation conditions developed for BIA derivatization in yeast will likely improve de novo synthesis as well (Box 2) [48]. Further strain engineering will be necessary for competitive titers of de novo BIA synthesis in yeast.

**Synthesis and Derivatization of Morphinan Alkaloids in S. cerevisiae**

Synthesis of naturally-occurring morphinan alkaloids proceeds through (R)-reticuline (Figure 2) [49,50]. The recent identification of the epimerase that catalyzes the conversion of (S)-reticuline to (R)-reticuline [18,51] marks the complete characterization of all the genes involved in morphinan biosynthesis in planta since the first isolation of morphine from opium poppy in 1806 [10]. Parts of the morphine pathway have been reconstituted in S cerevisiae by supplementation of intermediates. This year, the synthesis of opioids from simple carbon sources in a microbial system was achieved [52].

**Derivatization of Morphinans from Supplemented Precursors**

Thebaine is a primary feedstock for the chemical synthesis of naturally-occurring and semi-synthetic opioids [50]. As a proof of concept, S. cerevisiae expressing morphinan synthesis genes has been used to provide a microbial alternative to chemical derivatization of thebaine [53]. Synthesis of any single morphinan, and in particular morphine, from thebaine is an engineering challenge owing to the complex array of products that can be generated from a small number of enzymes. Morphine synthesis from thebaine requires the co-expression of three enzymes (Figure 2). Each enzyme has broad substrate specificity [14,54,55] and the reactions can occur in multiple orders. Two of these orders can result in morphine synthesis (pink and purple pathways in Figure 2) while other orders result in the synthesis of side-products (e.g., neopine and 14-hydroxycodeine). Spontaneous rearrangements (within the pink pathway in Figure 2) add another level of complexity because some are productive for morphine synthesis while others are not. In short, the array of morphinans produced is entirely dependent on the relative rates of enzyme activities and spontaneous reactions (Box 1).
Conversion of thebaine to morphine by yeast expressing the necessary enzymes was limited to 1.5%, with another 12.7% of thebaine being converted to intermediates and side-products, mainly neopine and 14-hydroxcodeine (Table 3) [53]. If flux travels through neopinone, these side-products will be difficult to avoid because neopine is generated when codeinone reductase (COR) activity outcompetes a spontaneous reaction, while 14-hydroxcodeine is generated when spontaneous reactions outcompete COR activity (Figure 2). Favoring synthesis through oripavine (purple pathway in Figure 2) is likely to reduce the number of side-products in morphine synthesis (Box 1). To achieve the reported 1.5% conversion of thebaine to morphine, some metabolic engineering strategies to favor synthesis of codeinone have already been employed. High-yield synthesis of morphine in microbes, although possible, will require the development of innovative strategies (see Box 1 for examples) to funnel the carbon flux towards morphine.

By contrast, the right combination of enzymes and spontaneous reactions can prove to be very effective at generating a particular morphinan of interest. For example, a pathway to hydrocodone and oxycodone was developed using genes isolated from a soil bacterium growing on industrial poppy waste (Figure 2, blue pathway) [53]. Without COR expression, many of the side-products of morphine synthesis were avoided, and almost half of supplemented thebaine was converted to the intended products (Table 3). Avoiding particular enzymes with high promiscuity, or identifying/engineering enzymes with desired substrate specificities, may prove to be a more general strategy for morphinan and/or BIA synthesis in vivo. For example, hydromorphone synthesis through neopinone (yellow pathway in Figure 2) resulted in only 0.4% hydromorphone (Table 3), whereas a hypothetical pathway through oripavine (indicated in green in Figure 2) could reduce side-products substantially because it avoids COR (Figure 2).

Thebaine itself has been derivatized from the upstream pathway intermediate (R)-reticuline in S. cerevisiae expressing the appropriate enzymes (Figure 2) [52,56]. Another spontaneous reaction presents an engineering problem unique to this portion of the pathway: the intermediate salutaridinol-7-O-acetate spontaneously rearranges to thebaine at pH 8–9, but rearranges to an undesired side-product at pH 6–7 (Figure 2) [57]. To address this issue, a two-step fermentation system was used in which yeast biomass was allowed to accumulate and subsequently switched to pH-buffered media supplemented with either (R)-reticuline or salutaridinol. The highest production of thebaine was observed at alkaline pHs (pH 8.5–9) [56]. This study highlights pH-adaptable fermentation conditions as an additional challenge for BIA synthesis in yeast (Box 2).

**Discovery of Reticuline Epimerase Enables De Novo Synthesis of Opiates**

Until recently, the fermentation of opiates from simple sugars was not possible owing to the inability to produce (R)-reticuline in vivo. Initially, the in vivo production of (R,S)-reticuline from spontaneously condensed (R,S)-norlaudanosoline was proposed as a source of (R)-reticuline [45,58]. However, more recent reports demonstrate that only (S) enantiomers can be accepted by enzymes of the norlaudanosoline-to-reticuline pathway that have been assayed thus far [31,56]. While enzymes capable of accepting (R) enantiomers may exist [59], epimerization of (S)-reticuline to (R)-reticuline is currently crucial for opiate biosynthesis. The enzyme catalyzing this stereochemical conversion is reticuline epimerase (REP), a cytochrome P450-reductase fusion protein discovered this year [18,51,60]. REP activity has been demonstrated in vitro [18,51] and in vivo, bridging the upper glucose-to-(S)-reticuline and lower (R)-reticuline-to-opiate sections of the pathway [38,40,52,56]. Yeast strains capable of converting supplemented (S)-norlaudanosoline or endogenously synthesized (S)-norcoclaurine to thebaine and hydrocodone have now been engineered. While yields are currently low (Table 3), areas of pathway improvement have been highlighted. In yeast capable of converting (S)-norlaudanosoline to (R)-reticuline, total reticuline levels were 100-fold lower than yields in other engineered S. cerevisiae strains lacking REP (Table 3) [58,61]. This could be due to the promiscuity of REP, which has been
Table 3. Derivatization of BIA Backbones in Microbial Hosts

<table>
<thead>
<tr>
<th>Initial Compound</th>
<th>Heterologous Genes</th>
<th>Final Compound(s)</th>
<th>% Yield</th>
<th>Backbone</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reticuline 100 μM</td>
<td>SAS, CPR, SAR, SAT</td>
<td>Salutaridine, 15 μM Thebaine, 1 μM</td>
<td>15</td>
<td>1</td>
<td>Pro-morphinan Morphinan [56]</td>
</tr>
<tr>
<td>Thebaine 1000 μM</td>
<td>COR, T6ODM, CODM</td>
<td>Codeine, 27 μM Morphine, 15 μM Other opiates, 100 μM</td>
<td>2.7</td>
<td>1.5</td>
<td>10</td>
</tr>
<tr>
<td>Thebaine 1000 μM</td>
<td>T6ODM, MorB</td>
<td>Hydrocodone, 180 μM Oxycodone, 220 μM Other opiates, 30 μM</td>
<td>18</td>
<td>22</td>
<td>3</td>
</tr>
<tr>
<td>Norlaud 1000 μM</td>
<td>60MT, CNMT, 4’OMT, NMCH, REP, SAS, CPR, SAR, SAT</td>
<td>Thebaine, 0.2 μM</td>
<td>0.02</td>
<td></td>
<td>Morphinan [52]</td>
</tr>
<tr>
<td>Glucose 110 000 μM</td>
<td>BH4 pathway, TH, DDCC, NCS, 60MT, CNMT, NMCH, 4’OMT, REP, SAS, CPR, SAR, SAT</td>
<td>Thebaine, 0.02 μM</td>
<td>&lt;0.001%</td>
<td></td>
<td>Morphinan [52]</td>
</tr>
<tr>
<td>Glucose 110 000 μM</td>
<td>BH4 pathway, TH, DDCC, NCS, 60MT, CNMT, NMCH, 4’OMT, REP, SAS, CPR, SAR, SAT, T6ODM, morB</td>
<td>Hydrocodone, 0.001 μM</td>
<td>&lt;0.001%</td>
<td></td>
<td>Morphinan [52]</td>
</tr>
<tr>
<td>Reticuline 33 μM</td>
<td>BBE</td>
<td>Scoulerine, 25 μM</td>
<td>76</td>
<td></td>
<td>Protobberine [45]</td>
</tr>
<tr>
<td>Reticuline 33 μM</td>
<td>CTS, CNMT</td>
<td>Magnofluorine, 21 μM</td>
<td>64</td>
<td></td>
<td>Aporphine [45]</td>
</tr>
<tr>
<td>Norlaud 4000 μM</td>
<td>60MT, CNMT, 4’OMT</td>
<td>Norlaud, 455 μM</td>
<td>10</td>
<td></td>
<td>Benzyloisoquinoline [58]</td>
</tr>
<tr>
<td>Norlaud 4000 μM</td>
<td>60MT, CNMT, 4’OMT, BBE</td>
<td>Scoulerine, 160 μM</td>
<td>4</td>
<td></td>
<td>Protobberine [58]</td>
</tr>
<tr>
<td>Norlaud 4000 μM</td>
<td>60MT, CNMT, 4’OMT, BBE, CPR, SOMT, CAS</td>
<td>Canadine, 88 μM</td>
<td>2.2</td>
<td></td>
<td>Protobberine [58]</td>
</tr>
<tr>
<td>Norlaud 10 μM</td>
<td>60MT, CNMT, 4’OMT, BBE, CPR, SFS, SPS, TNMT, MSH, P6H</td>
<td>Dihydrosanguinarine, 0.15 μM</td>
<td>1.5</td>
<td></td>
<td>Benzophenanthridine [61]</td>
</tr>
<tr>
<td>Norlaud 2000 μM</td>
<td>60MT, CNMT, 4’OMT, BBE, CPR, SFS, SPS, TNMT, MSH, P6H</td>
<td>Sanguinarine, 0.24 μM</td>
<td>0.012</td>
<td></td>
<td>Benzophenanthridine [48]</td>
</tr>
</tbody>
</table>

Metabolite abbreviation: Norlaud, norlaudanosoline.

*Molar yield.

Yield calculated from glucose, involving multiple steps not included in other strains in this table.

*Reticuline concentration estimated based on closest available standard.

demonstrated to accept norlaudanosoline-to-reticuline pathway intermediates in vitro [18]. In vivo, not all available (S)-reticuline was converted to (R)-reticuline, indicating that there is room to improve REP activity as well. Promoting REP activity on (S)-reticuline while limiting its activity on pathway intermediates will be an engineering challenge for the future (Box 1).

Derivatization of Other BIA Alkaloids in S. cerevisiae

Present and Future Diversity of Backbone Synthesis

In addition to morphinans, other BIA scaffolds have been produced in yeast (Table 3). In combination with (S)-coclaurine-N-methyltransferase, corytuberine synthase was used to
generate the aporphines coryzuberine and magnoflorine from reticuline [45]. Alternatively, the protoberberine scoulerine can be synthesized from reticuline by the berberine bridge enzyme (Figure 1B) [45,48,58,61]. Scoulerine is a precursor of the protopine, benzophenanthridine, phthalideisoquinoline, and rhoeadine scaffolds. Of these, protopine and benzophenanthridines have been synthesized in yeast [48,61]. Some of the phthalideisoquinoline noscapine pathway has been reconstituted, with scoulerine being converted to the downstream protoberberine canadine via the expression of scoulerine O-methyltransferase and canadine synthase [58]. Most of the noscapine synthesis pathway has now been elucidated, raising the possibility of its reconstitution in yeast [13,62]. Bisbenzylisoquinoline scaffolds, too, can likely be achieved in vivo because the recently-identified reticuline epimerase can also epimerize the (R)-benzylisoquinoline enantiomer required for berbamurine synthesis (Box 1 and Figure 1B in Box 1). The synthesis of rhoeadines from protopines is still unknown, as are the enzymes responsible for synthesizing other backbones shown in Figure 1B. Enzymes responsible for the addition of functional groups to many of these structures are also unknown.

**BIA Derivatization in Microbes Requires Knowledge of Synthesis Pathways**

The diversity of backbones highlights the flexibility of microbial systems for BIA production, while the many cytochrome P450-catalyzed reactions point to yeast as an ideal host (Figure 1B). However, the successes and absences of BIA scaffold synthesis in microbes demonstrate the underlying need for information about the enzymes responsible for their production. As genetic techniques for microbe manipulation rapidly improve the turnaround time and throughput of strain development [42,63], a limiting factor in microbial BIA diversification will become pathway elucidation. Plant biologists have been the traditional source of knowledge through metabolite and transcript profiling of induced and mutagenized plants [64,65]. In addition, the emergence of publicly-available transcriptome databases such as the 1000 Plants and PhytoMetSyn collections [66,67], combined with advanced techniques in strain engineering, will accelerate the functional discovery of unknown genes and the reconstitution of complex synthetic pathways in microbes.

**Biosecurity of Opiate Production in Microbes**

When de novo synthesis of BIAs in yeast was first reported this year, a single step remained to be elucidated that prevented the synthesis of opiates directly from simple carbon sources [38]. Reflecting on this, Oye et al. accompanied their report with a list of engineering and policy recommendations that groups seeking to create opiates in microbes should consider incorporating into their practices. Recently, the final step in morphine synthesis has not only been revealed [18,51] but also successfully introduced into yeast to synthesize opioids from sugar [52]. Access to this strain is limited to those with approval from the US Drug Enforcement Administration (DEA), as recommended by Oye et al. While titers remain low, it has been proposed to incorporate biosafety features into the high opiate producing strains to add an extra layer of security to dissuade their theft and malicious use in the future. Suggested features could include (non-disclosed) methods of preventing growth of the strain outside its intended use, as well as methods of identifying strains that have been stolen, such as introducing unique DNA signatures, or ‘watermarks’, into the strain.

**Concluding Remarks**

While the opiates are perhaps the most famous members of the benzylisoquinoline alkaloid family, many other members have potential and realized pharmaceutical value. The scalability and flexibility of microbes has encouraged their development as factories for BIA synthesis. De novo synthesis of (S)-reticuline in E. coli, achieved in 2011, is currently at 287 mg/l and will continue to grow, while de novo synthesis in yeast has been added this year. Titers are currently far from industrially-viable levels, with challenges at all levels of pathway development from improving the intracellular availability of precursors to preventing promiscuity in the final steps of
a pathway. Nevertheless, the pace towards industrial microbial fermentation has been impressive, mirroring recent advances in synthetic biology and metabolic engineering, and providing optimism for enabling the microbial production of this large family of natural products.

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