CellPress

Special Issue: Industrial Biotechnology

Review

Microbial Factories for the Production of Benzylisoquinoline Alkaloids

Lauren Narcross,[1,2] Elena Fossati,[1,2] Leanne Bourgeois,[1,2] John E. Dueber,[3] and Vincent J.J. Martin[1,2,*]

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.

Trends

Both *Escherichia coli* and *Saccharomyces cerevisiae* have been engineered to convert a simple carbon source such as glucose to complex BIAs.

The variety of BIA scaffolds synthesized in microbial hosts continues to increase, now encompassing benyliosquinolines, aporphines, protoberberines, protopines, benzophenanthridines, pro-morphinans, and morphinans.

Key challenges for future work have been identified, including pathway bottlenecks and the generation of side-products from promiscuous enzymes.

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

[1]Department of Biology, Concordia University, Montréal, Québec H4B 1R6, Canada
[2]Centre for Structural and Functional Genomics, Concordia University, Montréal, Québec H4B 1R6, Canada
[3]Department of Bioengineering, University of California, Berkeley, Berkeley, CA 94704, USA

*Correspondence: vincent.martin@concordia.ca (V.J.J. Martin).

 http://dx.doi.org/10.1016/j.tibtech.2015.12.005

Table 1. Diversity of BIAs in Drug Development

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

[a]Synthetic or semi-synthetic BIAs.

supply [11]. 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-hydroxyphenylacetaldehyde (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.

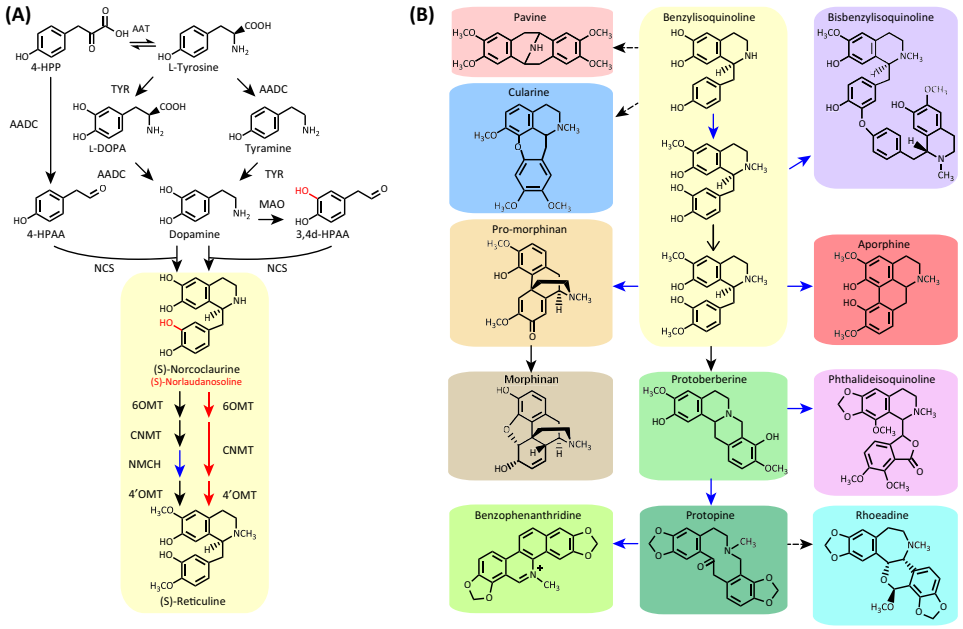
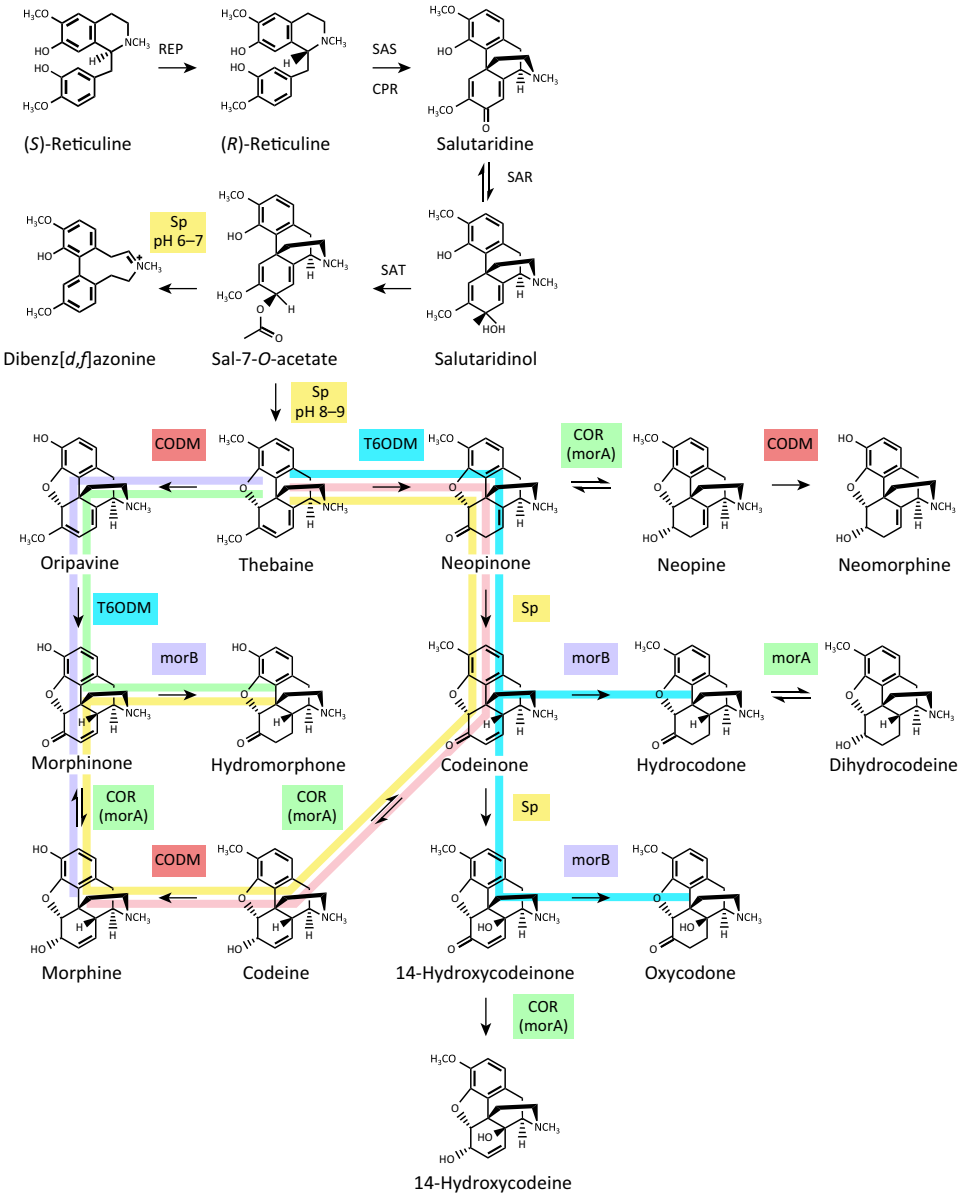


Figure 1. Microbial Synthesis of BIAs and BIA diversity. (A) 4-HPP is the microbial precursor to the substrates of norcoclaurine/norlaudanosoline condensation. Dopamine and 4-HPAA condense to form norcoclaurine (black), while dopamine and 3,4-dHPAA condense to form norlaudanosoline (red). The extra hydroxyl group on 3,4-dHPAA and norlaudanosoline is indicated in red, and reactions that accept norlaudanosoline 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, coclaurine *N*-methyltransferase; NCS, norcoclaurine synthase; NMCH, *N*-methylcoclaurine 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



Trends in Biotechnology

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* morphinone reductase (morB, purple), codeinone 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, salutardinol 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 IA) 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 [45]. 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 IB) [48,58], have improved yield in BIA synthesis pathways. Enzyme scaffolding could also push flux through the intended pathway (Figure IC). Sequestration of promiscuous enzymes into other organelles [53] or separate engineered microbes [37] has improved synthesis of BIAs (Figure IE). Alternatively, temporal control at the level of transcriptional regulation could allow buildup of the desired intermediate before the expression of a promiscuous enzyme (Figure IE).

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 IF). 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 [62]. This raises the possibility of re-engineering pathways to include blocking steps to avoid promiscuous enzymes, followed by later removal of the group (Figure IF), a method commonly used in synthetic organic chemistry.

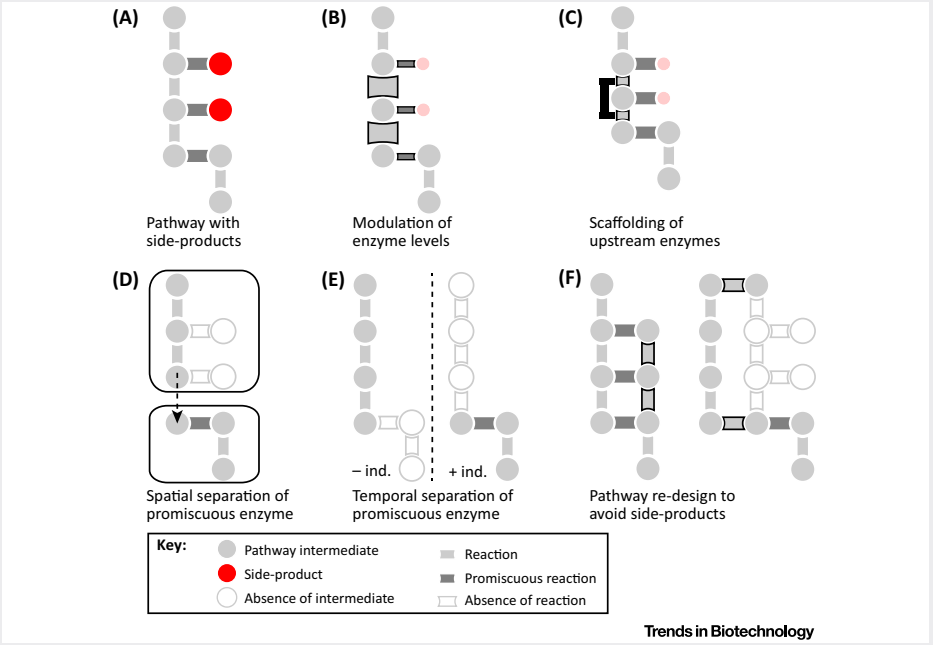


Figure I. Strategies for the Reduction of Side-Products in Pathways with Promiscuous Enzymes. (A) An initial pathway that results in the accumulation of side-products. (B) DNA copy number, ribosome binding site (RBS) strength, and promoter strength can be adjusted to improve flux by modulating enzyme expression (thinner pipe, less enzyme; fatter pipe, more enzyme; small red dot, less side-product). (C) Enzyme scaffolding (black line and shorter pipes) 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 tetrahydrobiopterin (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 BH_4 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 AAs 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

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 norlaudanosoline [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 norcoclaurine/norlaudanosoline (Figure 1A), is frequently used as a readout for *de novo* synthesis of norcoclaurine/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 BIAs 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

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

[a]Molar yield.
[b]Dopamine quantified in supernatant of strains expressing no downstream enzymes.
[c]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 K_M 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-hydroxycodeine (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-hydroxycodeine 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 salutaridine. 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

Initial Compound	Heterologous Genes	Final Compound(s)	% Yield[a]	Backbone	Ref.
Reticuline 100 μM	SAS, CPR, SAR, SAT	Salutaridine, 15 μM Thebaine, 1 μM	15 1	Pro-morphinan Morphinan	[56]
Thebaine 1000 μM	COR, T6ODM, CODM	Codeine, 27 μM Morphine, 15 μM Other opiates, 100 μM	2.7 1.5 10	Morphinan	[53]
Thebaine 1000 μM	T6ODM, MorB	Hydrocodone, 180 μM Oxycodone, 220 μM Other opiates, 30 μM	18 22 3	Morphinan	[53]
Norlaud 1000 μM	6OMT, CNMT, 4′OMT, NMCH, REP, SAS, CPR, SAR, SAT	Thebaine, 0.2 μM	0.02	Morphinan	[52]
Glucose 110 000 μM	BH4 pathway, TH, DODC, NCS, 6OMT, CNMT, NMCH, 4′OMT, REP, SAS, CPR, SAR, SAT	Thebaine, 0.02 μM	<0.001%[b]	Morphinan	[52]
Glucose 110 000 μM	BH4 pathway, TH, DODC, NCS, 6OMT, CNMT, NMCH, 4′OMT, REP, SAS, CPR, SAR, SAT, T6DOM, morB	Hydrocodone, 0.001 μM	<0.001%[b]	Morphinan	[52]
Reticuline 33 μM	BBE	Scoulerine, 25 μM	76	Protoberberine	[45]
Reticuline 33 μM	CTS, CNMT	Magnofluorine, 21 μM	64	Aporphine	[45]
Norlaud 4000 μM	6OMT, CNMT, 4′OMT	Reticuline[c], 455 μM	10	Benzylisoquinoline	[58]
Norlaud 4000 μM	6OMT, CNMT, 4′OMT, BBE	Scoulerine, 160 μM	4	Protoberberine	[58]
Norlaud 4000 μM	6OMT, CNMT, 4′OMT, BBE, CPR, SOMT, CAS	Canadine, 88 μM	2.2	Protoberberine	[58]
Norlaud 10 μM	6OMT, CNMT, 4′OMT, BBE, CPR, CFS, SPS, TNMT, MSH, P6H	Dihydrosanguinarine, 0.15 μM	1.5	Benzophenanthridine	[61]
Norlaud 2000 μM	6OMT, CNMT, 4′OMT, BBE, CPR, SPS, CFS, TNMT, MSH, P6H	Sanguinarine, 0.24 μM	0.012	Benzophenanthridine	[48]

Metabolite abbreviation: Norlaud, norlaudanosoline.
[a]Molar yield.
[b]Yield calculated from glucose, involving multiple steps not included in other strains in this table.
[c]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 corytuberine 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 berbamunine synthesis (Box 1 and Figure IB 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

Outstanding Questions

What factors limit norcoclaurine synthesis in yeast? With only 0.4% of endogenously produced dopamine being converted to norcoclaurine, increasing the yields of this committed step is crucial for developing yeast as an industrially-relevant host for BIA synthesis. Similarly, although dopamine-to-norcoclaurine conversion is currently 15% in *E. coli*, there is room for improvement.

Enzyme promiscuity in BIA synthesis pathways reduces the yields of BIAs produced in microbial hosts. To what extent can this be improved by metabolic engineering?

One of the advantages of synthesizing complex natural products such as BIAs in a microbial host is the flexibility to produce a vast array of different end-products. Much of the work in reconstituting BIA synthesis pathways has been focused on the synthesis of the key branch-point reticuline and its derivation into different BIA scaffolds. How can pathway elucidation *in planta* and *in silico* be combined with engineering efforts in microbes to expand the number of BIAs that can be made?

While the production of opiates in a microbial host provides alternatives to current production strategies, it also raises questions about the long-term possibility of illegitimate use. Is there a need for biosecurity measures to be implemented in opiate-producing strains and, if so, what should they be?

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.

Acknowledgments

This work was financially supported by a Discovery grant from the Natural Sciences and Engineering Research Council of Canada (NSERC). V.J.J.M. is supported by a Concordia University Research Chair and L.N. is supported by a Fonds pour la Formation à la Recherche dans l'Industrie et dans l'Agriculture (FQRNT) DE Doctoral Research Scholarship for Foreign Students.

References

1. Li, J.W-H. and Vederas, J.C. (2009) Drug discovery and natural products: end of an era or an endless frontier? *Science* 325, 161–165
2. Harvey, A.L. (2007) Natural products as a screening resource. *Curr. Opin. Chem. Biol.* 11, 480–484
3. David, B. *et al.* (2014) The pharmaceutical industry and natural products: historical status and new trends. *Phytochem. Rev.* 14, 299–315
4. Harvey, A.L. (2008) Natural products in drug discovery. *Drug Discov. Today* 13, 894–901
5. Lahlou, M. (2013) The success of natural products in drug discovery. *Pharmacol. Pharm.* 4, 17–31
6. Cordell, G.A. (2011) Sustainable medicines and global health care. *Planta Med.* 77, 1129–1138
7. Rathbone, D.A. and Bruce, N.C. (2002) Microbial transformation of alkaloids. *Curr. Opin. Microbiol.* 5, 274–281
8. Paddon, C.J. and Keasling, J.D. (2014) Semi-synthetic artemisinin: a model for the use of synthetic biology in pharmaceutical development. *Nat. Rev. Microbiol.* 12, 355–367
9. Pickens, L.B. *et al.* (2014) Metabolic engineering for the production of natural products. *Annu. Rev. Chem. Biomol. Eng.* 2, 211–236
10. Hagel, J.M. and Facchini, P.J. (2013) Benzylisoquinoline alkaloid metabolism: a century of discovery and a brave new world. *Plant Cell Physiol.* 54, 647–672
11. Cordell, G.A. *et al.* (2001) The potential of alkaloids in drug discovery. *Phyther. Res.* 15, 183–205
12. Stadler, R. *et al.* (1989) (S)-norcoclaurine is the central intermediate in benzylisoquinoline alkaloid biosynthesis. *Phytochemistry* 28, 1083–1086
13. Winzer, T. *et al.* (2012) A *Papaver somniferum* 10-gene cluster for synthesis of the anticancer alkaloid noscapine. *Science* 336, 1704–1708
14. Farrow, S.C. and Facchini, P.J. (2013) Dioxygenases catalyze O-demethylation and O O-demethylenation with widespread roles in benzylisoquinoline alkaloid metabolism in opium poppy. *J. Biol. Chem.* 288, 28997–29012
15. Takemura, T. *et al.* (2013) Molecular cloning and characterization of a cytochrome P450 in sanguinarine biosynthesis from *Eschscholzia californica* cells. *Phytochemistry* 91, 100–108
16. Ruff, B.M. *et al.* (2012) Biocatalytic production of tetrahydroisoquinolines. *Tetrahedron Lett.* 53, 1071–1074
17. Liscombe, D.K. and Facchini, P.J. (2007) Molecular cloning and characterization of tetrahydroprotoberberine cis-N-methyltransferase, an enzyme involved in alkaloid biosynthesis in opium poppy. *J. Biol. Chem.* 282, 14741–14751
18. Farrow, S.C. *et al.* (2015) Stereochemical inversion of (S)-reticuline by a cytochrome P450 fusion in opium poppy. *Nat. Chem. Biol.* 9, 728–732
19. Pittard, J. and Yang, J. (2008) Biosynthesis of the aromatic amino acids. *EcoSal Plus* 3, 3.6.1.8
20. Juminaga, D. *et al.* (2012) Modular engineering of L-tyrosine production in *Escherichia coli*. *Appl. Environ. Microbiol.* 78, 89–98
21. Gold, N.D. *et al.* (2015) Metabolic engineering of a tyrosine-overproducing yeast platform using targeted metabolomics. *Microb. Cell Fact.* 14, 1–16
22. McKenna, R. *et al.* (2014) Rational and combinatorial approaches to engineering styrene production by *Saccharomyces cerevisiae*. *Microb. Cell Fact.* 13, 1–12
23. Patnaik, R. *et al.* (2008) Tyrosine production by recombinant *Escherichia coli*: fermentation optimization and recovery. *Biotechnol. Bioeng.* 99, 741–752
24. Rodriguez, A. *et al.* (2015) Establishment of a yeast platform strain for production of p-coumaric acid through metabolic engineering of aromatic amino acid biosynthesis. *Metab. Eng.* 31, 181–188
25. Westfall, P.J. *et al.* (2012) Production of amorphadiene in yeast, and its conversion to dihydroartemisinic acid, precursor to the antimalarial agent artemisinin. *Proc. Natl. Acad. Sci. U.S.A.* 109, E111–E118
26. Kunjapur, A.M. *et al.* (2014) Synthesis and accumulation of aromatic aldehydes in an engineered strain of *Escherichia coli*. *J. Am. Chem. Soc.* 136, 11644–11654
27. Hazelwood, L.A. *et al.* (2008) The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. *Appl. Environ. Microbiol.* 74, 2259–2266
28. Kunjapur, A.M. and Prather, K.L.J. (2015) Microbial engineering for aldehyde synthesis. *Appl. Environ. Microbiol.* 81, 1892–1901
29. Rodriguez, G.M. and Atsumi, S. (2014) Toward aldehyde and alkane production by removing aldehyde reductase activity in *Escherichia coli*. *Metab. Eng.* 25, 227–237
30. Hawkins, K. (2009) *Metabolic Engineering of Saccharomyces cerevisiae for the Production of Benzylisoquinoline Alklaloids,* (PhD Thesis, California Institute of Technology), CaltechTHESIS
31. Nakagawa, A. *et al.* (2011) A bacterial platform for fermentative production of plant alkaloids. *Nat. Commun.* 2, 1–8
32. Cushing, M.L. (1948) The oxidation of catechol-type substrates by tyrosinase. *J. Am. Chem. Soc.* 70, 1184–1187
33. Prieto, M.A. *et al.* (1993) Characterization of an *Escherichia coli* aromatic hydroxylase with a broad substrate range. *J. Bacteriol.* 175, 2162–2167
34. Claus, H. and Decker, H. (2006) Bacterial tyrosinases. *Syst. Appl. Microbiol.* 29, 3–14
35. Hatlestad, G.J. *et al.* (2012) The beet R locus encodes a new cytochrome P450 required for red betalain production. *Nat. Genet.* 44, 816–820
36. Daubner, S.C. *et al.* (2011) Tyrosine hydroxylase and regulation of dopamine synthesis. *Arch. Biochem. Biophys.* 508, 1–12
37. Nakagawa, A. *et al.* (2014) (*R*,S)-tetrahydropapaveroline production by stepwise fermentation using engineered *Escherichia coli*. *Sci. Rep.* 4, 6695
38. DeLoache, W.C. *et al.* (2015) An enzyme-coupled biosensor enables (S)-reticuline production in yeast from glucose. *Nat. Chem. Biol.* 11, 465–471
39. Ehrenworth, A.M. *et al.* (2015) Pterin-dependent mono-oxidation for the microbial synthesis of a modified monoterpene indole alkaloid. *ACS Synth. Biol.* Published online July 27, 2015. http://dx.doi.org/10.1021/acssynbio.5b00025
40. Trenchard, I.J. *et al.* (2015) *De novo* production of the key branch point benzylisoquinoline alkaloid reticuline in yeast. *Metab. Eng.* 31, 74–83
41. Koopman, F. *et al.* (2012) *De novo* production of the flavonoid naringenin in engineered *Saccharomyces cerevisiae*. *Microb. Cell Fact.* 11, 1–15
42. Horwitz, A.A. *et al.* (2015) Efficient multiplexed integration of synergistic alleles and metabolic pathways in yeasts via CRISPR-Cas. *Cell Syst.* 1, 88–96

43. Stöckigt, J. *et al.* (2011) The Pictet–Spengler reaction in nature and in organic chemistry. *Angew. Chem. Int. Ed.* 50, 8538–8564
44. Minami, H. *et al.* (2007) Functional analysis of norcoclaurine synthase in *Coptis japonica*. *J. Biol. Chem.* 282, 6274–6282
45. Minami, H. *et al.* (2008) Microbial production of plant benzylisoquinoline alkaloids. *Proc. Natl. Acad. Sci. U.S.A.* 105, 7393–7938
46. Kim, J-S. *et al.* (2013) Improvement of reticuline productivity from dopamine by using engineered *Escherichia coli*. *Biosci. Biotechnol. Biochem.* 77, 2166–2188
47. Lichman, B.R. *et al.* (2015) 'Dopamine-first' mechanism enables the rational engineering of the norcoclaurine synthase aldehyde activity profile. *FEBS J.* 282, 1137–1151
48. Trenchard, I.J. and Smolke, C.D. (2015) Engineering strategies for the fermentative production of plant alkaloids in yeast. *Metab. Eng.* 30, 96–104
49. Ziegler, J. *et al.* (2006) Comparative transcript and alkaloid profiling in *Papaver* species identifies a short chain dehydrogenase/reductase involved in morphine biosynthesis. *Plant J.* 48, 177–192
50. Rinner, U. and Hudlicky, T. (2012) Synthesis of morphine alkaloids and derivatives. *Top. Curr. Chem.* 309, 33–66
51. Winzer, T. *et al.* (2015) Morphinan biosynthesis in opium poppy requires a P450-oxidoreductase fusion protein. *Science* 349, 309–312
52. Galanie, S. *et al.* (2015) Complete biosynthesis of opioids in yeast. *Science* 349, 1095–1100
53. Thodey, K. *et al.* (2014) A microbial biomanufacturing platform for natural and semisynthetic opioids. *Nat. Chem. Biol.* 10, 1–10
54. Unterlinner, B. *et al.* (1999) Molecular cloning and functional expression of codeinone reductase: the penultimate enzyme in morphine biosynthesis in the opium poppy *Papaver somniferum*. *Plant J.* 18, 465–475
55. Hagel, J.M. *et al.* (2010) Dioxygenases catalyze the O-demethylation steps of morphine biosynthesis in opium poppy. *J. Biol. Chem.* 6, 273–275
56. Fossati, E. *et al.* (2015) Synthesis of morphinan alkaloids in *Saccharomyces cerevisiae*. *PLoS ONE* 10, e0124459
57. Theuns, H.G. *et al.* (1984) Neodihydrothebaine and bractazonine, two dibenz[d,f]azonine alkaloids of *Papaver bracteatum*. *Phytochemistry* 23, 1157–1166
58. Hawkins, K.M. and Smolke, C.D. (2008) Production of benzylisoquinoline alkaloids in *Saccharomyces cerevisiae*. *Nat. Chem. Biol.* 4, 564–573
59. Beaudoin, G.A.W. and Facchini, P.J. (2014) Benzylisoquinoline alkaloid biosynthesis in opium poppy. *Planta* 240, 19–32
60. Beaudoin, G.A.W. (2015) *Characterization of Oxidative Enzymes Involved in the Biosynthesis of Benzylisoquinoline Alkaloids in Opium Poppy (Papaver somniferum),* (PhD thesis, University of Calgary), University of Calgary
61. Fossati, E. *et al.* (2014) Reconstitution of a 10-gene pathway for synthesis of the plant alkaloid dihydrosanguinarine in *Saccharomyces cerevisiae*. *Nat. Commun.* 5, 1–11
62. Dang, T-T.T. *et al.* (2015) Acetylation serves as a protective group in noscapine biosynthesis in opium poppy. *Nat. Chem. Biol.* 11, 104–106
63. Jakočiūnas, T. *et al.* (2015) Multiplex metabolic pathway engineering using CRISPR/Cas9 in *Saccharomyces cerevisiae*. *Metab. Eng.* 28, 213–222
64. Dang, T-T.T. *et al.* (2012) Biochemical genomics for gene discovery in benzylisoquinoline alkaloid biosynthesis in opium poppy and related species. *Methods Enzymol.* 515, 231–266
65. Desgagné-Penix, I. and Facchini, P.J. (2012) Systematic silencing of benzylisoquinoline alkaloid biosynthetic genes reveals the major route to papaverine in opium poppy. *Plant J.* 72, 331–344
66. Matasci, N. *et al.* (2014) Data access for the 1,000 Plants (1KP) project. *Gigascience* 3, 1–10
67. Facchini, P.J. *et al.* (2012) Synthetic biosystems for the production of high-value plant metabolites. *Trends Biotechnol.* 30, 127–131
68. Singla, D. *et al.* (2010) BIAdb: a curated database of benzylisoquinoline alkaloids. *BMC Pharmacol.* 10, 1–8
69. Peking Union Medical College Hospital (2011) *Pharmacokinetics and Pharmacodynamics of Higenamine in Chinese Healthy Subjects* (ClinicalTrials.gov Identifier NCT01451229), ClinicalTrials.gov
70. Bloomer, R. *et al.* (2015) Clinical safety assessment of oral higenamine supplementation in healthy, young men. *Hum. Exp. Toxicol.* 34, 1–11
71. Law, V. *et al.* (2013) DrugBank 4.0: Shedding new light on drug metabolism. *Nucleic Acids Res.* 42, D1091–D1097
72. Amirkia, V. and Heinrich, M. (2014) Alkaloids as drug leads – a predictive structural and biodiversity-based analysis. *Phytochem. Lett.* 10, xlviii–liii
73. Bowman, W.C. (2006) Neuromuscular block. *Br. J. Pharmacol.* 147, S277–S286
74. Frampton, J.E. and McTavish, D. (1993) Mivacurium. A review of its pharmacology and therapeutic potential in general anaesthesia. *Drugs* 45, 1066–1089
75. Cougar Biotechnology (2011) *A Study of Noscapine HCl (CB3304) in Patients with Relapsed or Refractory Multiple Myeloma* (ClinicalTrials.gov Identifier NCT00912899), ClinicalTrials.gov
76. Kang, H. *et al.* (2015) Glaucine inhibits breast cancer cell migration and invasion by inhibiting MMP-9 gene expression through the suppression of NF-κB activation. *Mol. Cell. Biochem.* 403, 85–94
77. Shanghai Jiao Tong University School of Medicine (2007) *Efficacy and Safety of Berberine in the Treatment of Diabetes with Dyslipidemia* (ClinicalTrials.gov Identifier NCT00462046), ClinicalTrials.gov
78. Peng, L. *et al.* (2015) Antibacterial activity and mechanism of berberine against *Streptococcus agalactiae*. *Int. J. Clin. Exp. Pathol.* 8, 5217–5223
79. Zha, W. *et al.* (2010) Berberine inhibits HIV protease inhibitor-induced inflammatory response by modulating ER stress signaling pathways in murine macrophages. *PLoS ONE* 5, 2–9
80. Meade, J.A. *et al.* (2015) (−)-Stepholidine is a potent pan-dopamine receptor antagonist of both G protein- and β-arrestin-mediated signaling. *Psychopharmacology (Berl.)* 232, 917–930
81. Food and Drug Administration (2003) Oral health care drug products for over-the-counter human use; antigingivitis/antiplaque drug products; establishment of a monograph. *Fed. Regist.* 68, 1–57
82. Nam, H. *et al.* (2012) Network context and selection in the evolution to enzyme specificity. *Science* 337, 1101–1104
83. Fischbach, M.A. and Clardy, J. (2007) One pathway, many products. *Nat. Chem. Biol.* 3, 353–355
84. Shin, J.H. *et al.* (2013) Production of bulk chemicals via novel metabolic pathways in microorganisms. *Biotechnol. Adv.* 31, 925–935
85. Aharoni, A. *et al.* (2005) The 'evolvability' of promiscuous protein functions. *Nat. Genet.* 37, 73–76
86. Yoshikuni, Y. *et al.* (2006) Designed divergent evolution of enzyme function. *Nature* 440, 1078–1082
87. Jones, J.A. *et al.* (2015) Metabolic pathway balancing and its role in the production of biofuels and chemicals. *Curr. Opin. Biotechnol.* 33, 52–59
88. Furubayashi, M. *et al.* (2015) A highly selective biosynthetic pathway to non-natural C50 carotenoids assembled from moderately selective enzymes. *Nat. Commun.* 6, 1–10
89. Martin, V. et al. Valorbec, Société en Commandite. Method of making a benzylisoquinoline alkaloid (BIA) metabolite, enzymes therefore, WO/2015/103711
90. Smolke, C.D. *et al.* The Board of Trustees of the Leland Stanford Junior University. Benzylisoquinoline alkaloids (bia) producing microbes, and methods of making and using the same, US20140273109 A1
91. Tang and Xueming *et al.* (2009) Microbial conversion of glycerol to 1,3-propanediol by an engineered strain of *Escherichia coli*. *Appl. Environ. Microbiol.* 75, 1628–1634