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Biocatalysts from alkaloid producing plants

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Metabolic pathways leading to benzyloisoquinoline and monoterpene indole alkaloids in plants are revealing remarkable new reactions. Understanding of the enzymes involved in alkaloid biosynthesis provides access to a variety of applications in biocatalysis and bioengineering. In chemo-enzymatic settings, plant biocatalysts can transform medically important scaffolds. Additionally, synthetic biologists are taking alkaloid pathways as templates to assemble pathways in microorganisms that are tailored to the needs of medicinal chemistry. In light of these many recent discoveries, it is expected that plants will continue to be a source of novel biocatalysts for the foreseeable future.

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Current Opinion in Chemical Biology 2016, **31**:22–30

This review comes from a themed issue on **Biocatalysis and biotransformation**

Edited by **Dan S Tawfik** and **Wilfred A van der Donk**

For a complete overview see the [Issue](#) and the [Editorial](#)

Available online 13th January 2016

<http://dx.doi.org/10.1016/j.cbpa.2015.12.006>

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Introduction

The versatile secondary metabolism of plants has been a rich source for natural products throughout human history. While the darkest recesses of bacterial and fungal secondary metabolism are becoming illuminated at a rapid pace [1], biocatalysts from plants have been more difficult to track down. The last missing step in morphine biosynthesis has only been identified in 2015 [2^{**},3^{**},4^{**}], more than 200 years after the opiate was isolated by Sertürner. The vanillin synthase from vanilla pods has remained elusive until 2014 [5] although vanillin is an integral part of everyday life as a food ingredient and fragrance. The late steps of vinblastine and taxol biosynthesis, both widely used cancer drugs, currently remain a mystery, not for lack of effort. Discovery of these biosynthetic enzymes can facilitate production of naturally occurring, high-value compounds. Additionally, plants carry out chemistry that is not observed in other organisms, making these enzymes sought-after biocatalysts.

Pathway elucidation is straightforward when one gene exposes a region in the genome where all other genes of

the pathway of interest are clustered [6]. Gene clustering is a typical feature in microorganisms, but unfortunately, such clusters are found inconsistently in plants [7]. Hence, plant genes have been historically assigned to chemical transformations one at a time, a tedious process even when the chemistry is well understood from isotopic feeding and other biochemical studies [8]. If not genomically, plant pathway genes may functionally cluster, which has been revealed by transcriptome sequencing and co-expression analysis [9]. These techniques have enabled a series of important gene discoveries [3^{**},10,11^{*},12].

This review focuses on recently unravelled enzymatic transformations involved in alkaloid biosynthesis, primarily in Madagascar periwinkle (*Catharanthus roseus*) and opium poppy (*Papaver somniferum*), and their potential for synthetic biology and biocatalysis. The presented enzymes from plant secondary metabolism can be broadly classified into scaffold generating enzymes that change the connectivity of carbon–carbon bonds and tailoring enzymes that, for instance, oxidize, acylate, glycosylate, or methylate these scaffolds. However, the boundaries can be blurred between these two categories [13].

Scaffold formation

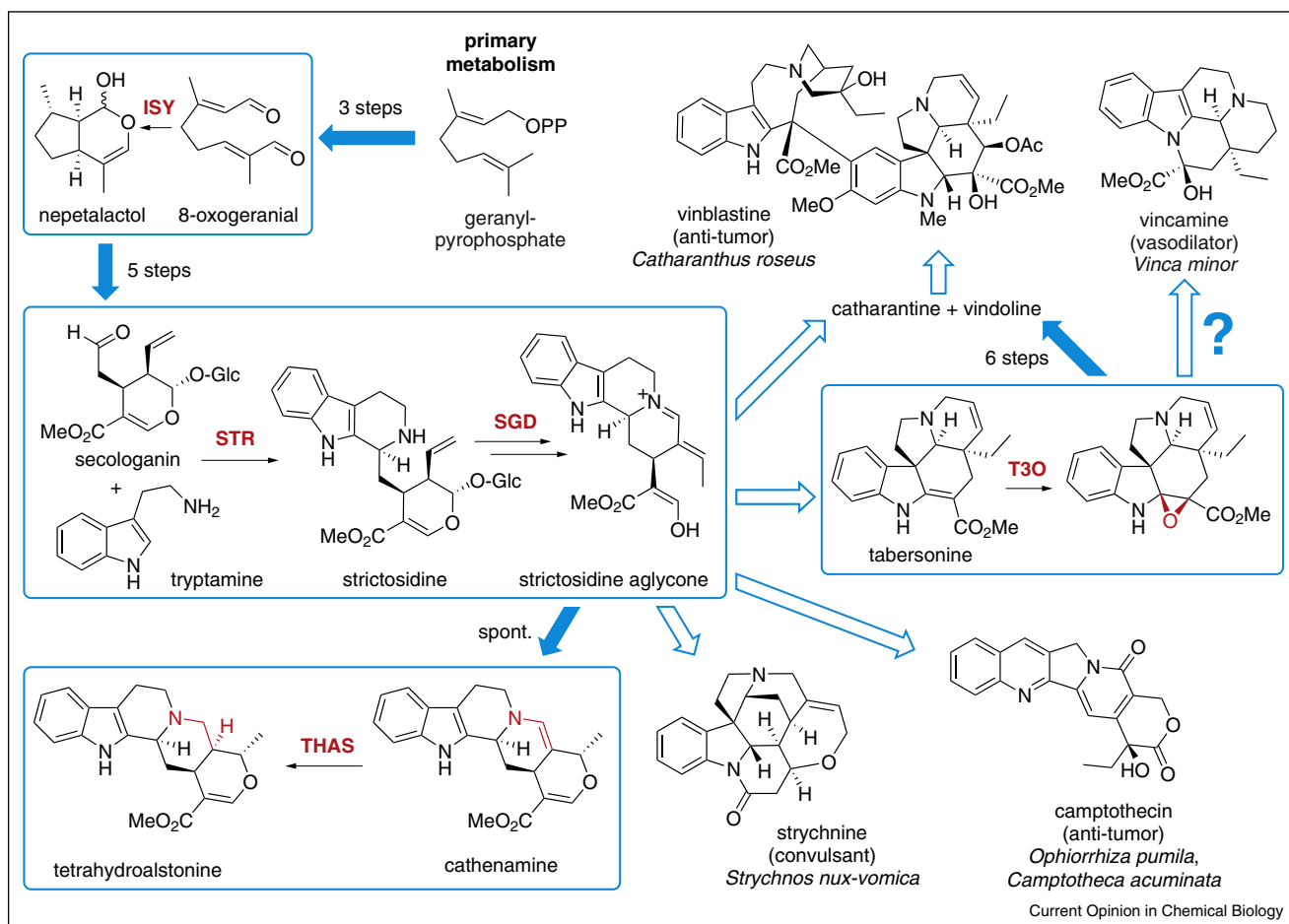
Iridoid synthase: adding a new twist to reduction

Synthetic chemists must envy the ease by which enzymes establish complex, cyclic molecular scaffolds from achiral precursors. A remarkable, reductive terpene cyclization [10,14] is involved in the reaction sequence linking the monoterpene geranyl pyrophosphate to the universal monoterpene indole alkaloid (MIA) precursor strictosidine (Figure 1). Iridoid synthase from *C. roseus* (CrISY) catalyzes reductive cyclization of the monoterpene 8-oxogeranial yielding the bicyclic iridoid nepetalactol in an NADPH dependent reaction. The reaction mechanism of CrISY is clearly distinct from the canonical, cationic mechanism of terpene cyclization. Although the atom connectivity suggested a hetero Diels–Alder reaction, experiments with substrate analogues were in better agreement with a reduction/Michael addition sequence [15]. The substrate 8-oxogeranial [16] and geranic acid, which resembles the expected enolate intermediate after hydride transfer, could be co-crystallized with the enzyme revealing the likely substrate binding mode and catalytic features [17]. The structure of CrISY can possibly inform future enzyme engineering efforts for the generation of alternative iridoid scaffolds.

Pictet–Spenglerases: versatile biocatalysts

Iridoids and other alkaloid precursors are often combined together by Pictet–Spenglerases in reactions that connect

Figure 1



Key reactions and products in MIA biosynthesis. The scaffold-forming enzymes ISY [10] and STR [18] catalyse key C–C bond forming reactions in MIA biosynthesis. THAS [32*] and T3O [11*,45] catalyse important redox-based tailoring steps. Un-filled arrows indicate steps that have not been elucidated on the enzyme level yet.

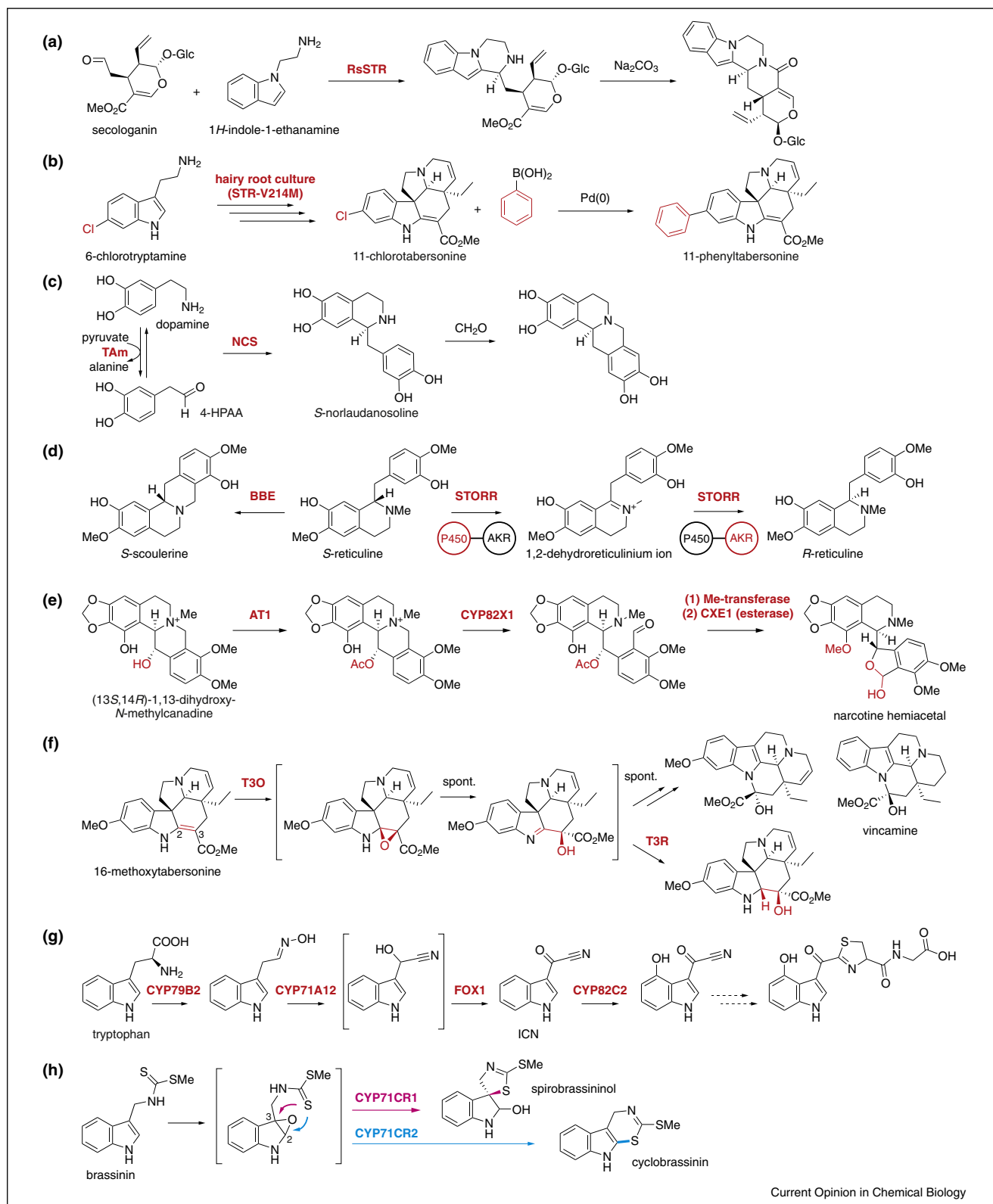
amines and carbonyls to alkaloid scaffolds [18]. The Pictet–Spengler condensation of tryptamine and the iridoid-derived aldehyde secologanin, for instance, is the key reaction in the biosynthesis of MIAs [19] (Figure 1). Strictosidine, the product of this reaction, is then further processed — often beyond recognition — to a myriad of downstream products, like strychnine, camptothecin, and vinblastine.

The Pictet–Spenglerase strictosidine synthase (STR) is a workhorse for biocatalytic applications [18] and shows interesting substrate promiscuity. Alternative indole alkaloid scaffolds were generated in a chemo-enzymatic approach that converts chemo-synthetic precursors with purified STR1 from *Rauwolfia serpentina* [20]. From 1*H*-indole-1-ethanamine and secologanin, STR1 synthesized a configurational isomer of strictosidine with a piperazino[1,2-*a*]indole scaffold, in which the indole ring is

‘flipped’ (Figure 2a). The engineering of STR also showcases how targeted mutations can remove roadblocks for biosynthetic diversification [21]. Hairy root cultures of *C. roseus* were transformed with halogenase genes *rebH* and *rebF* resulting in the production of halogenated tryptophan analogues *in vivo* that were envisioned to enter the MIA pathway. STR could be coerced into accepting 5- and 6-halogenated tryptamine by active-site mutation V214M [22,23]. As a result, halogenated alkaloids could be isolated from root tissue co-transformed with the halogenase genes and the mutated STR [21]. These halogenated alkaloid analogues were diversified even further by using the bromo-indole functionalities as handles for selective derivatization [23,24] (Figure 2b).

As part of a pathway leading to the important drug molecules codeine, morphine, noscapine, and berberine, the Pictet–Spenglerase norcochlorine synthase (NCS)

Figure 2



from benzyloquinoline alkaloid (BIA) biosynthesis has attracted considerable attention (Figure 3). In contrast to STR, the NCSs from *Thalictrum flavum* and *Coptis japonica* show exceptional substrate promiscuity for various aldehydes including a wide range of phenylacetaldehydes and even a few aliphatic aldehydes [25–27]. The aldehyde promiscuity of these Pictet–Spenglerases has been harnessed in biocatalytic approaches for the generation of chiral benzyloquinolines [25–27] and could be enhanced by mutagenesis [28]. By combining TfNCS with a transaminase for aldehyde precursor generation and reacting the initial isoquinoline product with formaldehyde, a tetrahydroprotoberberine alkaloid was formed in a one-pot reaction from dopamine [29] (Figure 2c). Altogether, these results augur well for future work on the diversification of MIAs and BIAs relying on natural or engineered substrate promiscuity of Pictet–Spenglerases.

Racemic benzyloquinolines resulting from non-enzymatic Pictet–Spengler or analogous reactions could be converted into optically pure material by berberine bridge enzyme (BBE) [30^{*}]. In the BIA pathway towards the antibiotic berberine (Figure 3), BBE closes a ring between an *N*-methylated isoquinoline and a phenol via FAD-dependent oxidation of the *N*-methyl group (Figure 2d). Gandomkar and co-workers observed that, although BBE was unable to cyclize synthetic *N*-ethyl derivatives of reticuline, they were efficiently dealkylated [30^{*}]. With a range of *N*-ethyl benzyloquinolines, BBE performed kinetic resolutions yielding dealkylated products in excellent enantiomeric excess (>98%).

Tailoring enzymes

THAS: deciding the fate of a labile intermediate

Little is known about the scaffold rearrangements in MIA biosynthesis that follow on from the Pictet–Spengler condensation catalysed by STR (Figure 1). A cascade of reactions is triggered by deglycosylation of strictosidine, which generates a chemically labile dialdehyde (strictosidine aglycone). In an intramolecular Schiff base formation, the tetracyclic strictosidine aglycone spontaneously closes to pentacyclic cathenamine and isomers [19,31]. At this biosynthetic branch point, only the path towards heteroyohimbine alkaloids has been elucidated: Tetrahydroalstonine synthase (THAS)

channels the metabolic flux towards this scaffold by reducing the iminium form of cathenamine [32^{*}] (Figure 1). By controlling the stereoconfiguration of the methyl group in cathenamine and of the incoming hydride, THAS selectively catalyzes formation of one out of four possible stereoisomers. Pending further mechanistic and structural investigations, the resemblance of THAS to sinapyl alcohol dehydrogenase [32^{*}] suggests a catalytic mechanism different from that of typical imine reductases [33].

Reticuline epimerization: an unprecedented fusion

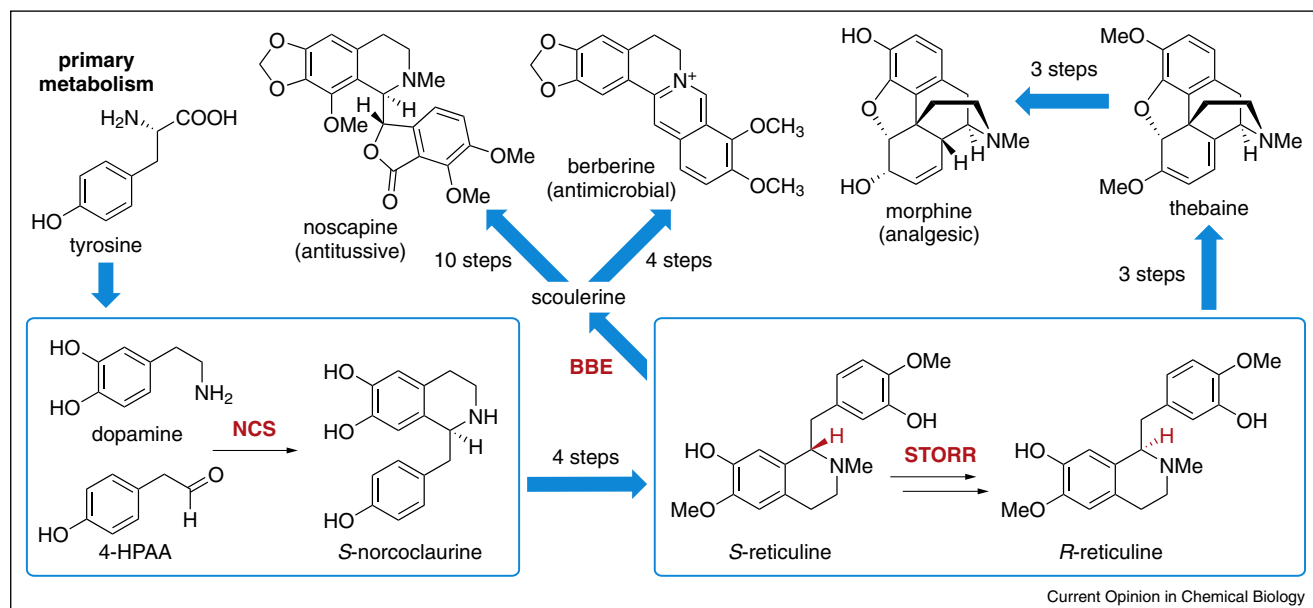
As with MIA biosynthesis, numerous dehydrogenases are involved in BIA metabolism and one of them performs an epimerization reaction that has eluded researchers for decades. Epimerization of reticuline — the last missing step in morphine biosynthesis — has recently been described by three independent groups led by Graham, Facchini, and Smolke [2^{**},3^{**},4^{**}] (Figures 2d, 3). While the Pictet–Spenglerase NCS generates the *S*-configuration, morphine has the *R*-configuration at this carbon. Therefore, an epimerization reaction is required in morphine biosynthesis. *S*- to *R*-reticuline epimerase (STORR) is an unprecedented fusion of an N-terminal cytochrome P450 domain that oxidizes the tertiary amine in *S*-reticuline to the corresponding iminium and a C-terminal reductase that performs an NADPH dependent reduction to the *R*-configuration (Figure 2d). When compared to other dehydrogenases in MIA metabolism, the reductase segment of STORR belongs to the aldo-keto reductase class, similar to codeinone reductase, whereas the BIA enzymes salutaridine reductase [34] and sanguinarine reductase [35] belong to the short-chain dehydrogenase (SDR) class. In the final step leading to the BIA noscapine, the newly discovered SDR noscapine synthase was shown to oxidize the narcotine hemiacetal to the lactone noscapine [36]. Interestingly, this enzyme was originally discovered as part of a 10 gene cluster in the opium poppy genome [37].

Noscapine synthesis: acetylation orchestrates oxidation

Earlier in noscapine synthesis in opium poppy, the action of three P450 oxidases is orchestrated by transient acetylation, reminiscent of protective group usage in organic synthesis [38^{*}]. P450 enzymes hydroxylate the noscapine precursor *S*-*N*-methylcanadine at two positions. The third hydroxylation only takes place when one of the other

(Figure 2 Legend) Recently discovered or engineered biochemical reactions from plants that are highlighted in this review. **(a)** STR from the MIA biosynthetic pathway can be used to make a novel piperazino[1,2-*a*]indole scaffold structure [20]. **(b)** Site-directed mutagenesis of STR allows a broader spectrum of substrates to be turned over, including 5 and 6 halogenated tryptamine analogues. These halogenated derivatives can be further derivatized by cross-coupling reactions [21–23]. **(c)** The Pictet–Spenglerase NCS from BIA biosynthesis can be coupled with formaldehyde treatment to yield a more complex alkaloid [29]. **(d)** *S*-Reticuline can enter two different routes. In berberine biosynthesis, BBE oxidatively closes the methylamine to a ring yielding *S*-scoulerine [30^{*}]. In morphine biosynthesis, an unprecedented fusion protein consisting of a P450 and a chalcone reductase is responsible for epimerization of the morphine biosynthetic intermediate reticuline [2^{**},3^{**},4^{**}]. **(e)** An acetyl group acts as a protecting group during noscapine biosynthesis [38^{*}]. **(f)** The P450 T3O performs a net-hydration either by generating an amine epoxide that rearranges to the imine alcohol, or forms the imine-alcohol directly [11^{*},45]. This reaction is integral to vinblastine biosynthesis, and also may be involved in the biosynthesis of vincamine. **(g)** *Arabidopsis* forms an unprecedented α -ketonitrile scaffold [46^{**}]. **(h)** In *Brassica*, P450s catalyse epoxide formation and subsequently control cyclization to form either the spirobrassinol or cyclobrassinol product [47^{*}].

Figure 3



Key reactions and products in BIA biosynthesis. The BIA pathway harbours numerous enzymes that can be exploited for biocatalysis purposes. Both the Pictet–Spenglerase NCS (Figure 2c) [25–29] and the flavoenzyme BBE [30^{*}] have been used in biocatalytic applications. The last missing step in morphine biosynthesis, STORR, has been recently elucidated (Figure 2d) [2^{**},3^{**},4^{**}].

hydroxyl groups is acetylated and yields a labile carbinoamine (Figure 2e). Then, opening of the carbinolamine triggers a skeletal rearrangement. The carbonyl group originating from the carbinolamine and the hydroxyl group liberated by deprotection of the acetyl group form a hemiacetal. Interestingly, there is no obvious chemical requirement for the presence of the acetyl group. It is tempting to speculate that acetylation/deacetylation may be conserved as a vestigial trait due to the stringent substrate specificity of these enzymes that is unlikely to change simultaneously [38^{*}].

P450s in *C. roseus*: hydroxylation and beyond

P450 enzymes also play important roles in the MIA pathway of *C. roseus*. On the way from geranyl-pyrophosphate to secologanin, 7-deoxyloganin-7-hydroxylase [39] and 7-deoxyloganin-7-hydroxylase introduce a hydroxyl group, and oxidize a methyl group to a carboxylate, respectively [40,41]. Several steps later, P450 enzymes T16H1, T16H2, [42] and T19H act on tabersonine [43] by introducing hydroxyl groups. Although the chemical transformations involved are not unusual, complete knowledge of these enzymes is crucial for pathway reconstitution approaches [41,44^{*}].

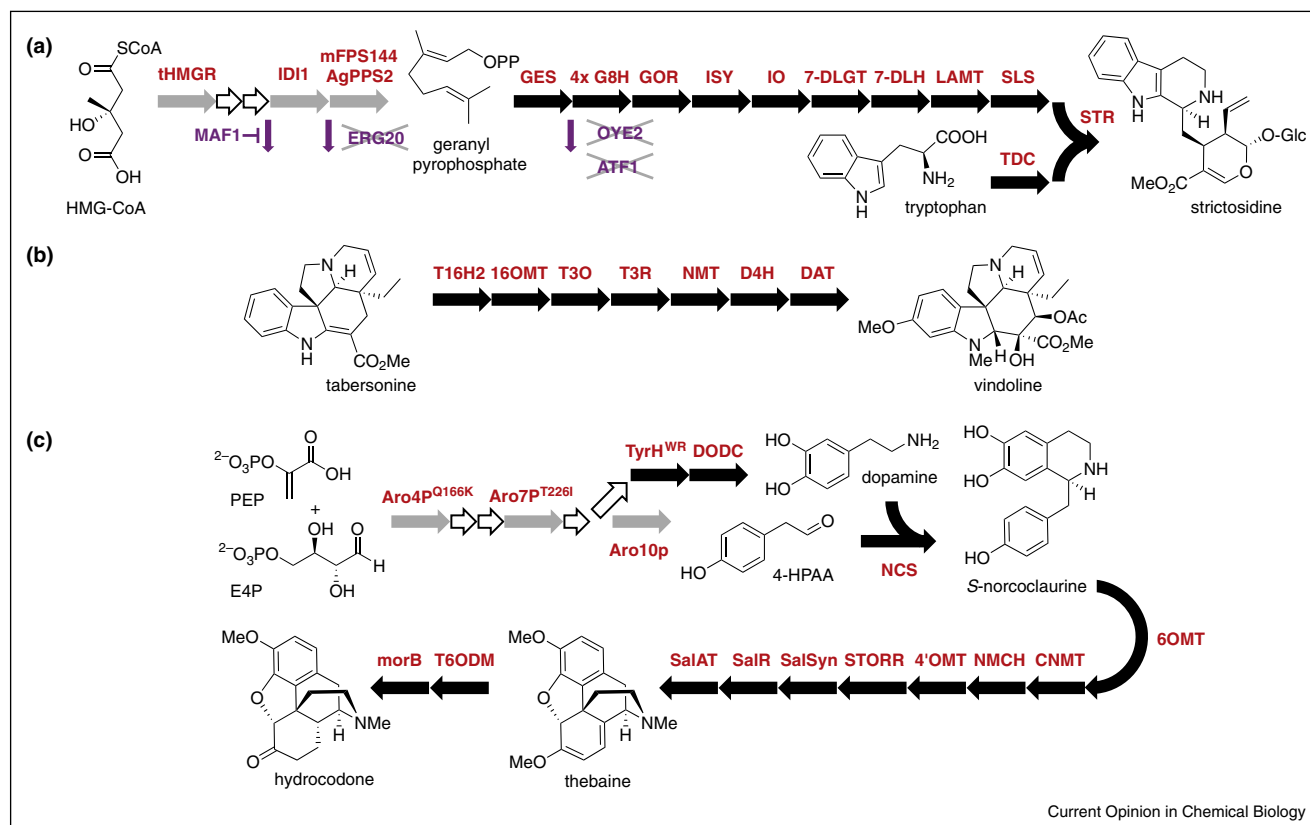
A transformation of 16-methoxy-tabersonine, in what seems to be a P450 catalysed hydration of an enamine double bond, has less chemical precedence and may play a role also in scaffold formation [11^{*},45] (Figure 2f). This transformation is apparently triggered by oxidation of the

enamine double bond to an epoxide by 16-methoxy-tabersonine 3-oxidase (T3O), independently discovered by the groups of O'Connor and de Luca. *In planta*, the amino epoxide likely opens to an imino alcohol that 16-methoxy-tabersonine-3-reductase (T3R) reduces to the amino alcohol (Figure 2f) [45]; alternatively, the enzyme may generate the imino alcohol directly [11^{*}]. Kellner *et al.* have proposed an intriguing hypothesis about an alternative, multistep rearrangement that takes place in the absence of the subsequent downstream reductase [45]. NMR spectra of an *in vitro* reaction conducted with T3O and 16-methoxy-tabersonine demonstrate that the product resembles vincamine, an alkaloid not present in *C. roseus* but in the related genus *Vinca* (Figure 2f). Future experiments are needed to show whether the T3O triggered rearrangement can indeed explain the biosynthesis of this additional vinca alkaloid scaffold.

Brassicaceae: new P450s in well-known plants

Co-expression analysis on the transcriptome level had a share in all P450 discoveries mentioned above. In combination with metabolomics studies, RNAseq analysis has aided not only in gene, but also in natural product discovery in a plant where new natural products may have been least expected [46^{**}]. *Arabidopsis* tissues under pathogen-stress revealed consistent upregulation of *CYP82C2*. When this gene was knocked out, the biosynthetic intermediate indole-3-carbonyl nitrile (ICN) accumulated (Figure 2g). *CYP82C2* apparently hydroxylates ICN at C4 of the indole ring. By using this enzyme as bait

Figure 4



Discovery of new plant-derived enzymes allows reconstitution of complex pathways in heterologous organisms such as *Nicotiana benthamiana* and *Saccharomyces cerevisiae*. Black arrows indicate newly introduced enzymatic steps, grey arrows indicate steps that are not new to the heterologous host but had to be enhanced by introducing additional genes, un-filled arrows indicate reactions that were not modified, and purple arrows indicate off-pathway reactions that were reduced by knocking-out or inhibiting host genes. Accessory enzymes like cytochrome P450 reductases were also introduced but are not shown. (a) The central MIA biosynthetic intermediate strictosidine has been produced in *S. cerevisiae* [44]. Reconstitution of the same pathway in *N. benthamiana* has proven fruitful for candidate verification [41]. (b) The late stage MIA intermediate vindoline, a direct precursor for the anti-cancer agent vinblastine, has been produced in seven steps from tabersonine in *S. cerevisiae* [11]. (c) Opiates from the morphine pathway have been produced *de novo* in *S. cerevisiae* [4,51,52,53].

for co-expression analysis, two P450s and a dehydrogenase were discovered that synthesize ICN from tryptophan via an oxime and a cyanohydrin (Figure 2g). The α -ketonitrile functional group of ICN is highly unusual in natural products.

Other indolic compounds occur in crucifers widely cultivated as food plants and their biosynthesis provides another example where P450s act beyond their usual scope of reactivity. The presence of spirobrassinin and cyclobrassinin in *Brassica rapa* has recently been traced back to the action of two P450 enzymes catalysing alternative *S*-heterocyclizations on brassinin [47] (Figure 2h). An epoxide introduced by the P450s at C2 and C3 of the indole ring is intramolecularly attacked by a dithiocarbamate moiety either at C2 or C3, yielding cyclobrassinin or spirobrassinin. Since the enzymes CYP71CR2 and CYP71CR1 almost exclusively yielded one or the other product under *in vitro* conditions, it can be assumed that

they catalyse regioselective cyclization in addition to epoxidation.

Pathway reconstitution

An incentive for elucidating the biosynthesis of plant natural products is not only the exploitation of individual plant biocatalysts, but also the assembly of entire metabolic pathways in new hosts. The industrial production of the plant terpenoid artemisinin for use as an antimalarial drug [48], which is partially conducted in engineered yeast, foreshadows a bright future for plant pathway reconstitutions [49]. Alkaloids, too, have been the target of pathway reconstitutions in inexpensively cultured microorganisms like yeast or in the efficiently transformable model plant *Nicotiana benthamiana* [50]. While yeast can provide scalable biosynthetic methods, pathway reconstitutions in *N. benthamiana* are useful to functionally verify gene candidates in an orthogonal plant host. The MIA precursor strictosidine, for instance, has been

produced in *N. benthamiana* in seven steps starting from the externally supplied intermediate iridodial thus validating several gene candidates [41]. The earlier part of the pathway starting from primary metabolites also functioned, but not in combination with the later enzymes. Although *Saccharomyces cerevisiae* seems even more challenging as a host, here, strictosidine production was successfully achieved by introducing 14 genes from the MIA pathway, seven accessory genes and three gene deletions [44*] (Figure 4a). Gene deletions helped to redirect the metabolic flux away from undesired off-pathway metabolites, for instance sesquiterpenes, to the desired target strictosidine.

Initial successes are promising, but reconstructed pathways have to become considerably more efficient and several genes need to be discovered before production of complex indole alkaloid drugs like vinblastine becomes feasible in microorganisms. Vindoline, a late precursor of vinblastine, has been biosynthesized in seven steps from the MIA tabersonine in yeast [11*] (Figure 4b) but missing genes between strictosidine and tabersonine obstruct the connection to primary metabolism. Pathway reconstruction of BIAs [4**,51,52,53*] is one step ahead since morphine biosynthesis has been fully elucidated (Figure 4c). Nevertheless, yields of thebaine or hydrocodone are only in the range of micrograms per litre and several orders of magnitude lower than necessary to compete with isolation from plants [4**]. In the future, metabolic bottlenecks may be removed by tuning transcriptional promoters, optimizing codon usage, blocking side reactions, upregulating the precursor synthesis, engineering the enzymes, or perhaps by laboratory evolution of strains.

Conclusion

Important gaps in our understanding of plant metabolism have been filled in recent years but even larger gaps remain. When fundamentally new chemistry is discovered in a thoroughly investigated model plant like *A. thaliana* [46**], this suggests that we are only scratching the surface of the metabolic complexity present in more than 200 000 species of flowering plants. With every newly discovered enzyme, gene discovery efforts contribute to a rapidly growing set of biocatalysts that can render chemical syntheses more efficient and sustainable. The impact of plant biocatalysts will likely be amplified when structural and mechanistic studies provide the framework for enzyme engineering that can significantly broaden their catalytic scope [54].

Acknowledgements

Work in the authors' lab was supported by the European Research Council (ERC R20359) and a BBSRC Institute Strategic Programme Grant (MET; BB/J004561/1). We gratefully acknowledge a SNF Early Postdoc Mobility Fellowship (to HK). We thank Dr Thu-Thuy T Dang for providing a photograph of *P. somniferum*.

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