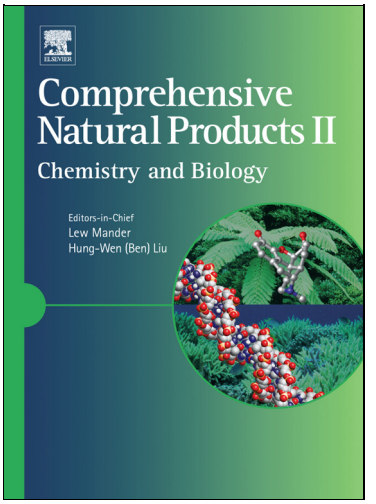

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1.25 Alkaloids

Sarah E. O'Connor, Massachusetts Institute of Technology, Cambridge, MA, USA

1.25.1 What Is an Alkaloid?

Alkaloids encompass an enormous class of approximately 12 000 natural products.[1] The principal requirement for classification as an alkaloid is the presence of a basic nitrogen atom at any position in the molecule, which does not include nitrogen in an amide or peptide bond. As implied by this exceptionally broad definition, the alkaloids form a group of structurally diverse and biogenically unrelated molecules. Most classes of natural products are composed of similar chemical structures, in which the same starting materials are assembled in related biosynthetic pathways. For example, all polyketides derive from acetate and propionate building blocks that undergo a series of Claisen condensation reactions.[2] However, no biochemical paradigm is centrally applied throughout alkaloid biosynthesis. Instead, the biosynthetic pathways of alkaloids are as diverse as the chemical structures found within this class of natural products.

Historical reasons account for this broad chemical definition of an alkaloid.[3] Prior to the nineteenth century, all compounds purified from plants – such as tartaric acid, oxalic acid, and tannins – exhibited acidic properties. However, alkaline material called potash extracted from burnt wood was later found to contain basic compounds of pharmacological interest. Meissner suggested in 1819 that these compounds be referred to as alkaloids, meaning a plant-derived substance that displays alkaline properties.[3]

In 1806, Friedrich Wilhelm Serturner, for the first time, isolated a pure compound that exhibited the same pharmacological sleep-inducing properties of the crude opium extract from which this compound was isolated. The discovery of this alkaloid compound, subsequently named morphine, played a key role in the development of what was to become the modern pharmaceutical industry, and the purification of many other pharmacologically important alkaloids such as strychnine, quinine, and caffeine rapidly followed suit (**Figure 1**).[3] Notably, accurate structural elucidation of the alkaloids was much more difficult. Many alkaloid structures remained unknown until well into the twentieth century when X-ray spectroscopy became widely available, and after organic chemistry had advanced to the point where these molecules could be produced synthetically.

Although plants were the first known source of alkaloid compounds, it is now known that fungi, bacteria, insects, and animals also produce a wide array of alkaloids. Therefore, the definition of alkaloids has been expanded to state "alkaloids are nitrogen-containing organic substances of natural origin with a greater or lesser degree of basic character".[3]

1.25.2 Classes of Alkaloids

Alkaloids are most commonly constructed from amino acid starting materials, although some purine-derived alkaloids are also known. The structural class of the alkaloid is typically defined by the substrate starting material. For example, tyrosine is used for the production of tetrahydroisoquinoline alkaloids (**Figure 2(a)**).

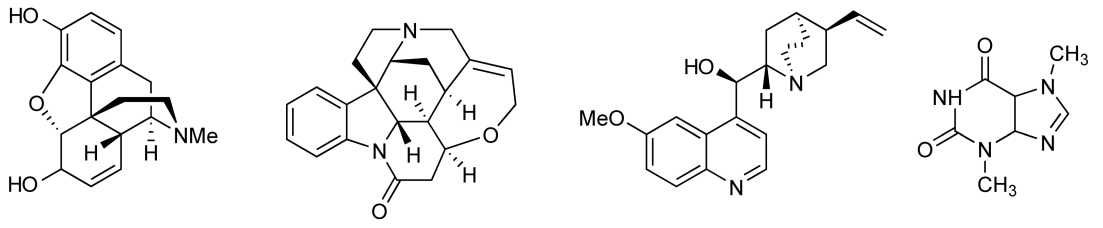


Figure 1 Pharmacologically important alkaloids discovered early in the history of alkaloids. Morphine, strychnine, quinine, and caffeine are shown.

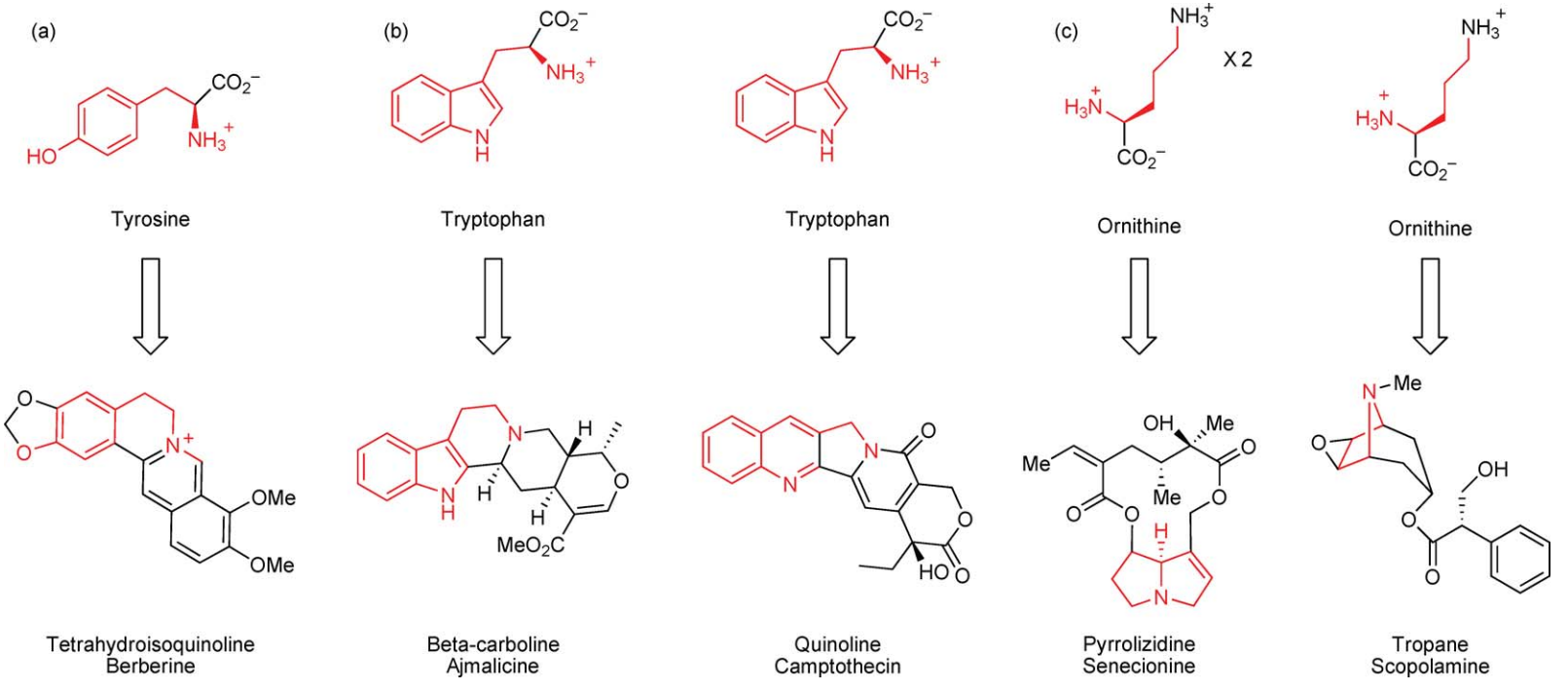
Tryptophan is the starting material for indole-containing alkaloids, such as alkaloids containing a β-carboline moiety (**Figure 2(b)**). Additionally, the indole of tryptophan can be modified to form the quinoline alkaloids (**Figure 2(b)**). Ornithine, a nonproteogenic amino acid derived from glutamate or arginine, is used to produce the pyrrolizidine- and tropane-type alkaloids (**Figure 2(c)**). Nicotinic acid can be used in combination with other amino acids to yield the nicotinic alkaloids (**Figure 2(d)**). Lysine, which contains one extra methylene group compared to ornithine, produces the structurally analogous piperidine, quinolizidine, and indolizidine alkaloids (**Figure 2(e)**). Known alkaloids derived from other amino acids are more rare. In addition to amino acid building blocks, a number of other nitrogen-containing starting materials can serve as alkaloid precursors. Anthranilic acid, a precursor to tryptophan, is used to produce quinazoline-, quinoline-, and acridine-type alkaloids (**Figure 2(f)**). A number of purine-derived alkaloids have also been isolated (**Figure 2(g)**). Finally, some alkaloids acquire the requisite basic nitrogen through transamination of an existing polyacetate or terpenoid framework. These compounds are referred to as pseudoalkaloids.

1.25.3 Function and Diversity of Alkaloids

The function of alkaloids is still not entirely clear. Although the pharmacological uses of many alkaloids are well defined, the specific roles that these compounds play in the producing organism are not well elucidated in most cases. At one point it was speculated that alkaloids were simply waste products derived from the degradation of primary metabolites. However, although the exact roles of many alkaloids remain poorly understood, these compounds are now believed to play an important ecological role, enabling the producing organism to defend itself and interact with its environment. In fact, although natural products are often termed secondary metabolites, the vital role that many natural products play in signaling and development renders the term 'secondary' a misnomer.

Most obviously, many alkaloids are toxic, so biosynthesis of these compounds provides a general defensive mechanism for the producing organism. For example, caffeine has been shown to act as an insecticide. Caffeine may act as a natural insecticide in plants. When the three *N*-methyltransferase genes involved in caffeine biosynthesis were overexpressed in tobacco, the resulting increase in caffeine production improved the tolerance of the plants to certain pests.[4] Occasionally, alkaloids that are present in animals are acquired by predation on a plant alkaloid producer. In one example, a moth species *Tyria jacobaeae* feeds on the plant *Senecio jacobaea*, which produces a number of pyrrolizidine alkaloids. The moth detoxifies the molecule by oxidizing the nitrogen to an N-oxide using a flavin-dependent monooxygenase (**Figure 3**). If the moth is ingested by a predator, the N-oxide is reduced in the gut of the predator where it is degraded to highly toxic pyrrole moieties. The regulation of natural products also suggests a defensive function for these compounds. Many natural products, including alkaloids, are upregulated under stressful conditions. For example, sterilized preparations of fungal cell wall extracts, called fungal elicitors, can be added to plant cell culture to increase the levels of alkaloid production in many plant species.

Nevertheless, it remains unclear why such a diversity of vastly different defensive, toxic molecules is required. This diversity is particularly apparent in the tetrahydroisoquinoline and monoterpene indole classes of alkaloids, where thousands of alkaloid products are generated from a single, central biosynthetic



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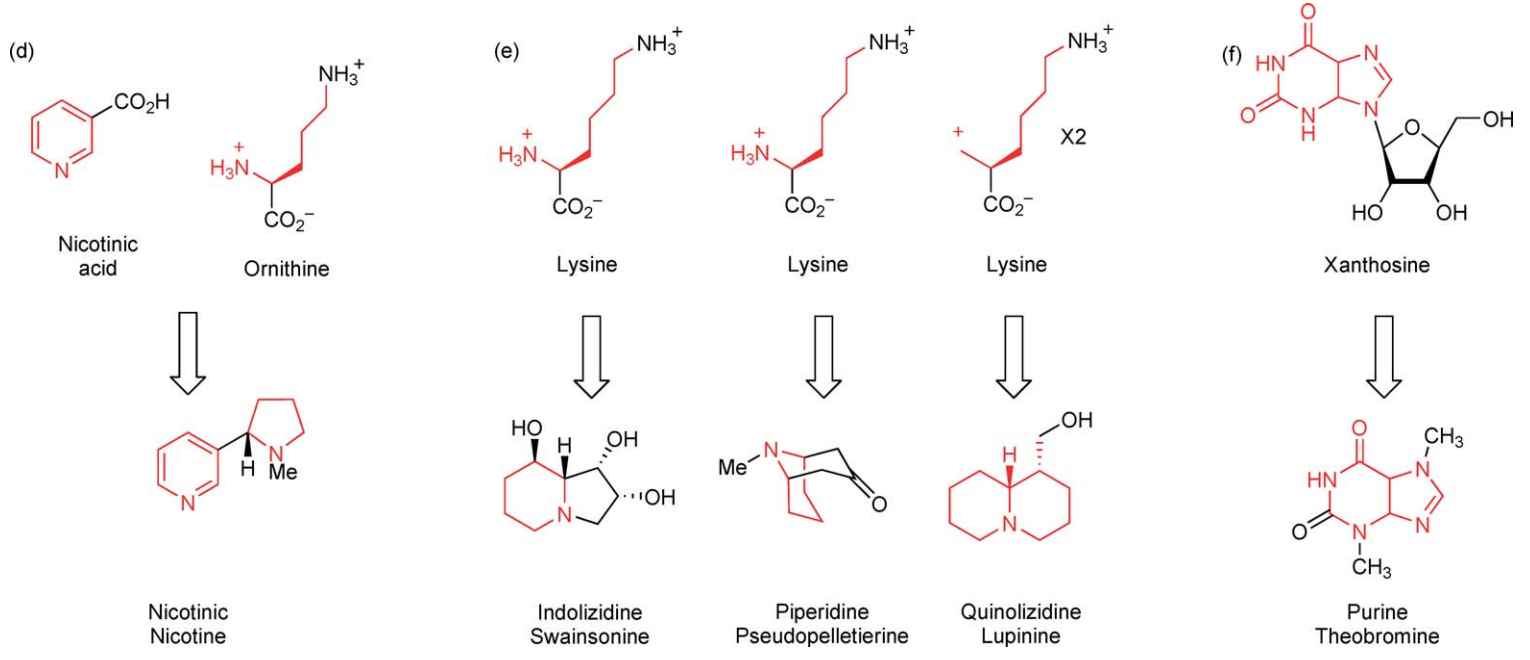

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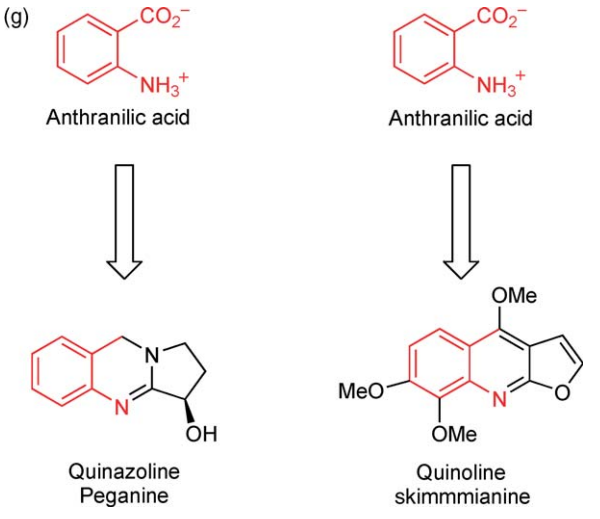
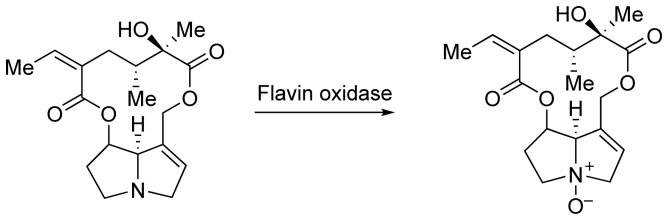


Figure 2 Representative members of the major structural classes of alkaloids. (a) Tyrosine-derived tetrahydroisoquinoline alkaloids. (b) Tryptophan-derived monoterpene indole alkaloids containing a β-carboline or quinoline moiety. (c) Ornithine-derived pyrrolizidine- and tropane-type alkaloids. (d) Nicotinic acid-derived alkaloid. (e) Lysine-derived piperdine, quinolizidine, and indolizidine alkaloids. (f) Anthranilic acid-derived quinazoline-, quinoline-, and acridine-type alkaloids. (g) Purine-derived alkaloid.



Sennecione Sennecione oxide

Figure 3 Oxidation of pyrrolizidine alkaloid for detoxification.

intermediate. A single medicinal plant known to produce these alkaloids typically generate 100 or more members of the alkaloid family. A recent commentary highlights that 'primary metabolic pathways are target-oriented', in other words, designed to produce a single, highly optimized molecule.[5] Many secondary or natural product pathways are, in contrast, 'diversity oriented'. Firn and Jones[6] hypothesized that since potent biological activity is a rare property for any molecule to have, "an organism needs the ability to make multiple molecules in order to hit upon the rare potent ones". In many species, natural products serve as an immune system of sorts. If the producing organism continuously evolves its capacity to generate new structures, the organism may be advantageously positioned for survival in its environment.

1.25.4 Strategies for Elucidating Alkaloid Biosynthesis

First and foremost, a mechanistic elucidation begins with the accurate structural characterization of the alkaloid product. Natural product structure elucidation posed a formidable challenge in the early days of alkaloid isolation. A variety of strategies have made this process much easier, namely, X-ray analysis and high-field 2D nuclear magnetic resonance (NMR) techniques.[7] Additionally, total synthesis of a reported complex alkaloid structure is a critical strategy for confirming the structural features of the natural product. A mechanistic elucidation of a natural product biosynthetic pathway typically begins by establishing which precursors are involved. Isotopically labeled (potential) precursors are fed to the producing organism, and the desired alkaloid product is then isolated. If the alkaloid contains the isotopic label, then it is clear that the precursor was used in the biosynthesis. Older experiments relied on radiolabeled precursors, since detection of radioactivity in the final product is sensitive and straightforward. However, the routine use of powerful, high-resolution mass spectrometry techniques has made the detection of safe, inexpensive stable isotopes such as hydrogen-2 (deuterium), carbon-13, and nitrogen-15 much more practical. After establishing the identity of the correct starting materials, strategic positioning of isotopic labels can be used to probe the mechanism of the transformations and structural rearrangements that occur along the biosynthetic route. These experiments, when placed in the context of previously known biochemical transformations, enable a series of logical enzymatic reactions to be proposed.

Identification of the enzymes, and the corresponding genes that encode them, constitutes the next level of pathway elucidation. How these genes are identified, or cloned, is dependent on the identity of the producing organism. Historically, alkaloid research has focused on plant-derived compounds. Plants are immobile and interact with their environment largely via the release of complex small molecules, so it is not surprising that this evolutionary pressure has resulted in the production of an extremely diverse array of natural products by plants. However, elucidating the genes of a plant pathway poses significant challenges. In contrast to microbes, the genes of plant pathways – with a few exceptions[8,9] – are not clustered on the genome, so each gene of a plant pathway must be discovered individually. Additionally, the genome sizes of medicinal plants are much larger (>1000 Mbp) than the typical natural product-producing bacteria (~8 Mbp), which makes finding and screening putative biosynthetic genes difficult. Although spectacular successes have been achieved in elucidating plant pathways, the challenges of plant biology have hindered the study of plant secondary metabolism and most plant-derived pathways remain incompletely elucidated at the genetic level. However, complex, higher plants frequently produce compounds that are not found in any known bacteria or fungi.

The majority of plant biosynthetic enzymes have been identified using a classical approach in which the enzyme of interest is purified from a crude plant lysate by standard protein chromatography.[10] Once a homogenous preparation of enzyme is prepared, partial protein sequence is obtained from the purified protein, which is then used to identify the gene encoding the desired enzyme. In another approach, if a guess can be made as to the type of enzyme involved in the desired transformation, a homology-based cloning strategy can be used.[11] Enzymes within a given class contain highly conserved regions in the protein sequence. Oligonucleotide primers complementary to these consensus sequences can be used to amplify gene candidates that can be screened for function. In a third approach called subtractive hybridization, cDNA from two types of tissue can be 'subtracted' from one another.[12] The genes in tissue that produces natural products at high levels can be compared with gene expression levels in tissue that produce low levels of alkaloid in question. The genes unique to each tissue type are readily obtained, and presumably, at least some of the genes that are unique to the alkaloid-producing tissue are involved in alkaloid biosynthesis. Finally, suppression (by RNAi)[13] or activation (by T-DNA tagging)[14] of large numbers of genes, followed by screening for changes in the levels of alkaloid production, can also be used to identify biosynthetic enzymes, provided that a fast screen or selection is available to interpret the phenotype of the transformed lines. Many plant alkaloid enzymes have been elucidated, but the plant alkaloids that have been studied in the most detail at the genetic level are the isoquinoline alkaloids,[1,15] the terpenoid indole alkaloids,[16] the tropane alkaloids,[17,18] and the purine alkaloids[19] (**Figure 4**).

Bacteria, whether terrestial or marine in habitat, typically export an arsenal of natural products involved in defense and signaling. Bacteria have much smaller genomes than plants, and the genes of bacterial natural product pathways tend to be organized in clusters, which makes identification of an entire metabolic pathway much more straightforward than in plants. Historically, bacteria have not been a rich source of alkaloids, although several complex alkaloid pathways have been recently discovered.[20,21] Undoubtedly, many more alkaloids will be discovered as the technical and financial barriers to sequence whole bacterial genomes continue to decrease, and more bacterial species are readily available for genetic analysis.

The study of microbe-derived natural products underwent a revolution in the 1980s as improved sequencing technologies allowed the rapid discovery of the genes that encode natural product biosynthetic pathways.[22] Since the genes of bacterial-derived pathways are relatively easy to identify, elucidation of these pathways is less dependent on isotopic labeling studies. Bioinformatic analysis of the genes in the cluster can provide clues as to the types of enzymatic transformations that take place. Although traditionally, the desired gene cluster is targeted and isolated in a cosmid vector for sequence analysis, it can now be cost-effective to simply sequence the entire genome of the producing strain and use bioinformatic analysis to search for candidate gene clusters in the genome sequence. After identification of the cluster, heterologous expression of candidate enzymes followed by *in vitro* biochemical assay of various combinations of these enzymes and substrates provide important information. Finally, if the producing organism can be genetically manipulated, targeted gene deletions can be made to definitively validate that the cluster in question is responsible for the production of the compound. Most notably, the prodiginines[23] and the indolocarbazoles[24–26] such as rebeccamycin, staurosporin, and violecin are prokaryotic alkaloids that have been the subject of several recent investigations. Benzodiazapines[27] and saframycins[28–30] have also been the subject of recent study (**Figure 5**).

Fungi, like bacteria, also produce a wealth of natural products. Eukaryotic fungal organisms generally have larger genomes and more complex life cycles, so, while fungi are still much simpler than higher plants they are considerably more complex than bacteria. It appears that the genes of many metabolic pathways are also clustered in fungal genomes, thereby greatly simplifying the study of fungal biosynthetic pathways. Nevertheless, fungal clusters are typically larger than prokaryotic clusters, and the genes often contain short introns. Several fungal alkaloids have also been partially elucidated at the genetic level. The ergot alkaloids[31] and the indole diterpenes[32] are two major classes of fungal alkaloids that have been studied extensively (**Figure 6**).

In addition to plants, bacteria, and fungi, many other organisms produce extraordinary alkaloid structures. Sponges as well as other multicelled marine organisms, insects, amphibians, and even mammals all produce complex alkaloid natural products.[33,34] However, in general, the biosynthetic pathways from these complex organisms are much less well characterized than the pathways from microbial and plant kingdoms. In many of these cases, the producing organisms are not viable in a laboratory environment, which complicates the elucidation of the biosynthetic mechanism. Additionally, genetic manipulation of many of these organisms is not yet developed.

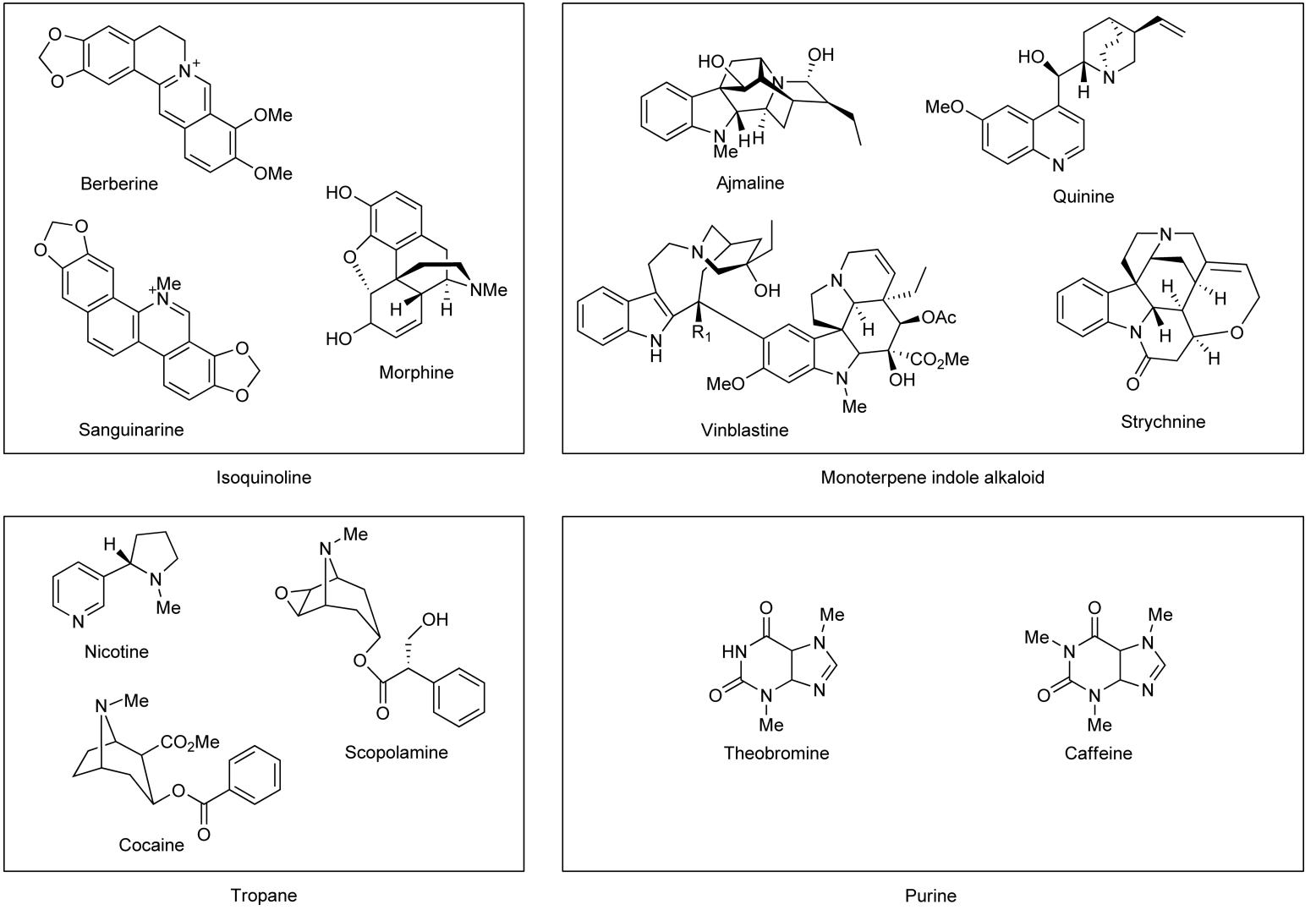


Figure 4 Representative plant-derived alkaloids from the tetrahydroisoquinoline, monoterpene indole, tropane, and purine classes.

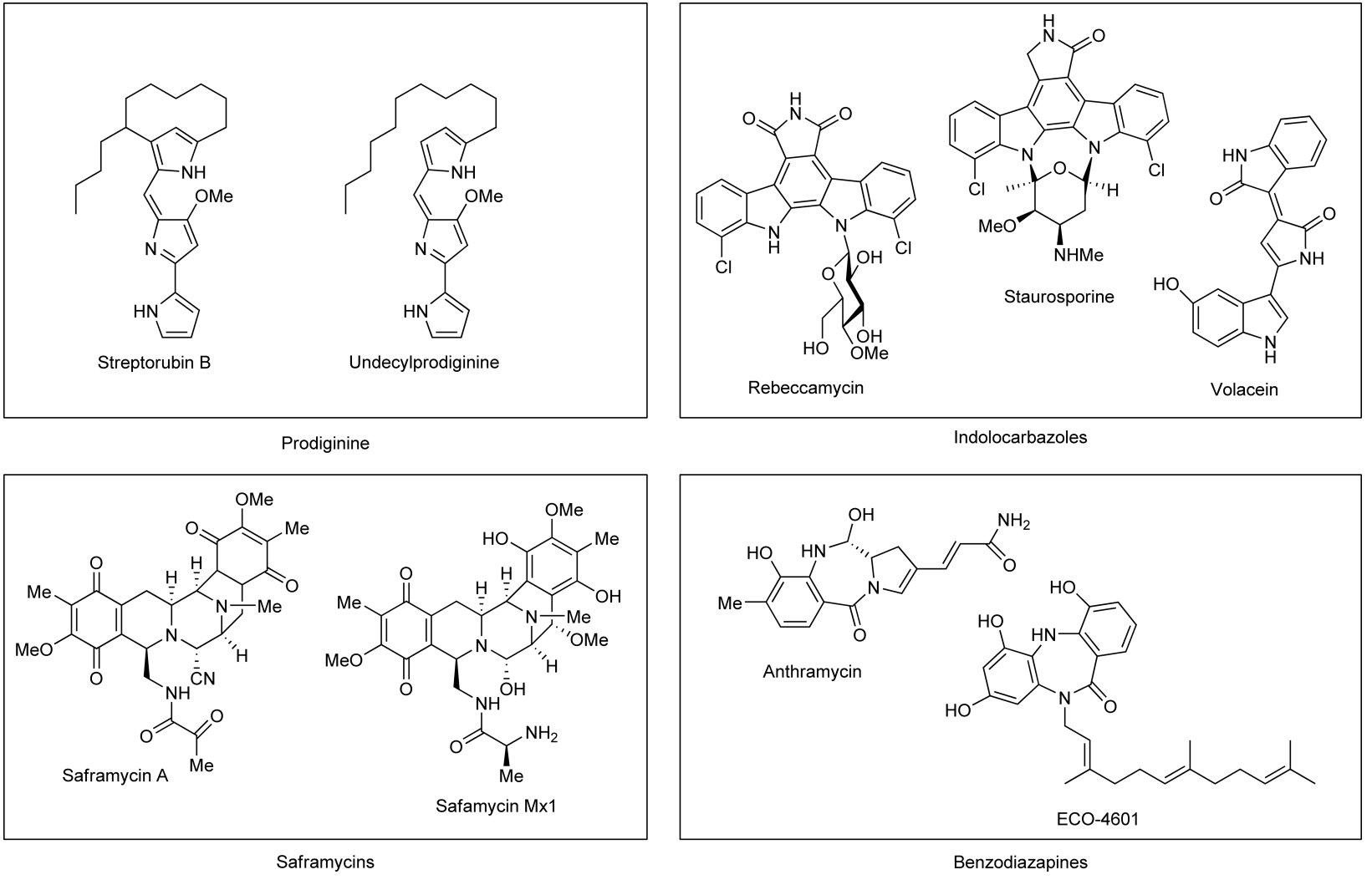


Figure 5 Representative prokaryote-derived alkaloids from the prodiginine, indolocarbazole, saframicin, and benzodiazapine classes.

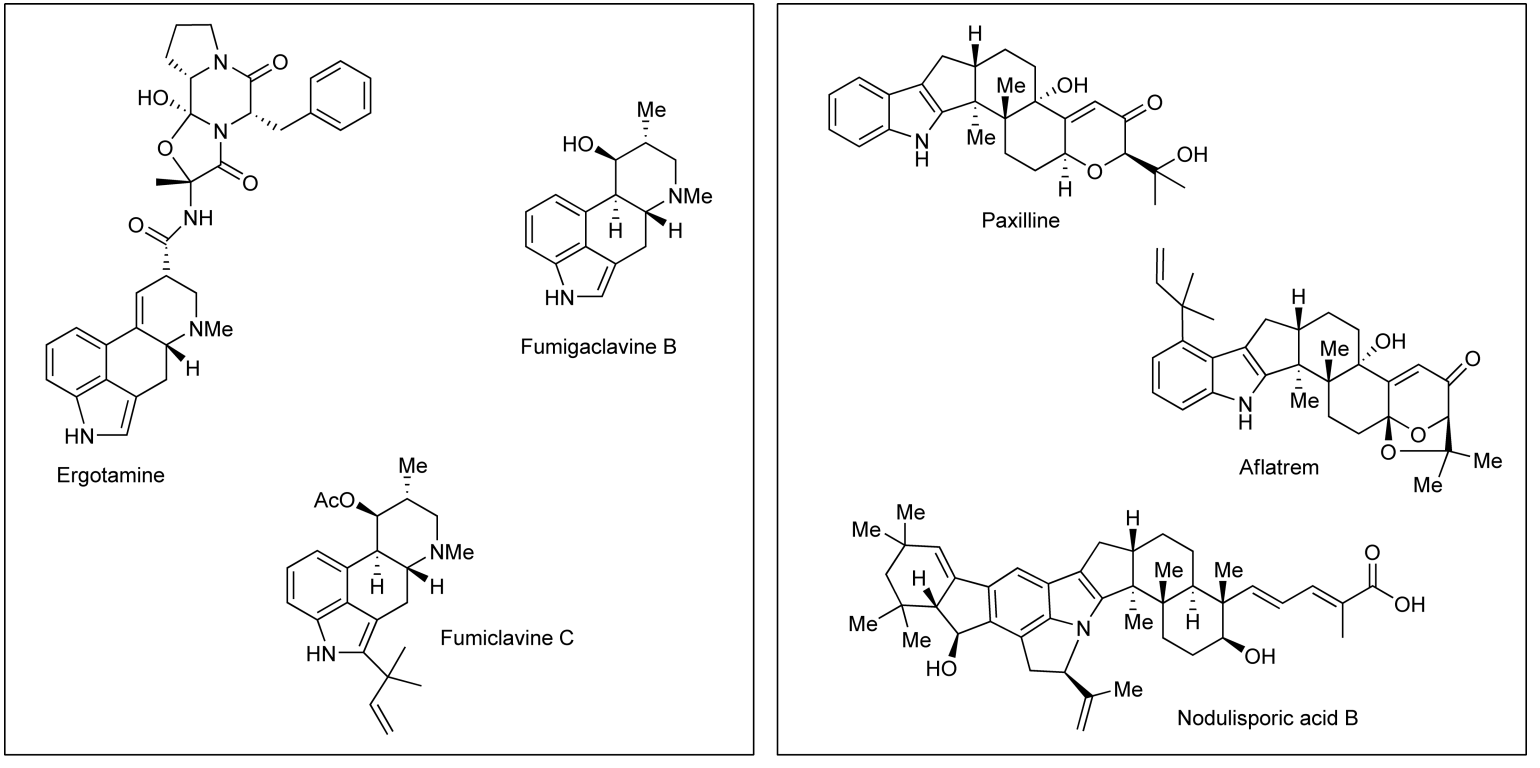


Figure 6 Representative fungal-derived alkaloids of the ergot and indole diterpene types.

A comprehensive discussion of all known alkaloid biosynthetic mechanisms extends well beyond the scope of this chapter. Here, we focus on the small number of plant-derived alkaloids for which genetic information regarding the biosynthetic pathway has been elucidated. Even within this limited subset of alkaloid structures, the structural diversity that is observed among the alkaloids becomes apparent, and the chemistry involved in these relatively few pathways is wide ranging.

1.25.5 Benzylisoquinoline Alkaloid Biosynthesis

The isoquinoline alkaloids include, most famously, the opiates morphine and codeine as well as the antibiotic berberine (**Figure 7**). Morphine and codeine are two of the most important analgesics used in medicine, and plants remain the main commercial source of the alkaloids.[35] Notably, development of plant cell cultures of *Eschscholzia californica*, *Papaver somniferum*, and *Coptis japonica* has aided in the isolation and cloning of many enzymes involved in the biosynthesis of isoquinoline alkaloids.[15]

Isoquinoline biosynthesis begins with the substrates dopamine and *p*-hydroxyphenylacetaldehyde to yield the central intermediate of this biosynthetic pathway, (*S*)-reticuline (**Figure 8**). Tyrosine is hydroxylated and decarboxylated to yield dopamine. Enzymes that catalyze the hydroxylation and decarboxylation steps in either order exist in the plant, and the predominant pathway for the formation of dopamine from tyrosine is not clear. The second substrate, *p*-hydroxyphenylacetaldehyde, is synthesized by transamination and decarboxylation of tyrosine.[36] Dopamine and *p*-hydroxyphenylacetaldehyde are coupled by the enzyme norcoclaurine synthase to form (*S*)-norcoclaurine. Two norcoclaurine synthases with completely unrelated sequences have been cloned (*Thalictrum flavum* and *C. japonica*) and heterologously expressed in *Escherichia coli*.[37,38] One shows homology to iron-dependent dioxygenases, whereas the other is homologous to a pathogenesis-related protein. Recent structural analysis of one of these enzymes has shed light onto the mechanism of this enzymatic transformation. Undoubtedly, future experiments will explain how two such widely divergent sequences can catalyze the same reaction.

One of the hydroxyl groups of (*S*)-norcoclaurine is methylated by an *S*-adenosyl methionine (SAM)-dependent *O*-methyltransferase to yield (*S*)-coclaurine. This enzyme has been cloned, and the heterologously expressed enzyme exhibited the expected activity.[39,40] The next biosynthetic intermediate is N-methylated to yield *N*-methylcoclaurine, an enzyme that has also been cloned.[41,42] *N*-Methylcoclaurine is then hydroxylated by a P-450-dependent enzyme (CYP80B), *N*-methylcoclaurine 3′-hydroxylase, that has been cloned.[43] One of the hydroxyl groups is methylated by the enzyme 3′-hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase (4′-OMT) to yield (*S*)-reticuline, the common biosynthetic intermediate for this pathway (**Figure 8**).[44–46] The biosynthetic pathway then diverges to yield the different structural classes of isoquinoline alkaloids.

In one major pathway, (*S*)-reticuline is converted to (*S*)-scoulerine by the action of a well-characterized flavin-dependent enzyme, berberine bridge enzyme (**Figure 9**). This enzyme has been cloned from several plant species,[47–49] and the mechanism of this enzyme has been studied extensively.[50,51] Notably, a structural analysis of this enzyme has been recently reported.[52] (*S*)-Scolerine is then methylated by scoulerine 9-*O*-methyltransferase to yield (*S*)-tetrahydrocolumbamine. Heterologous expression yielded an enzyme that had the expected substrate specificity.[53] The substrate-specific cytochrome P-450 oxidase canadine synthase that generates the methylene dioxy bridge of (*S*)-canadine has been cloned.[54] The final step of berberine

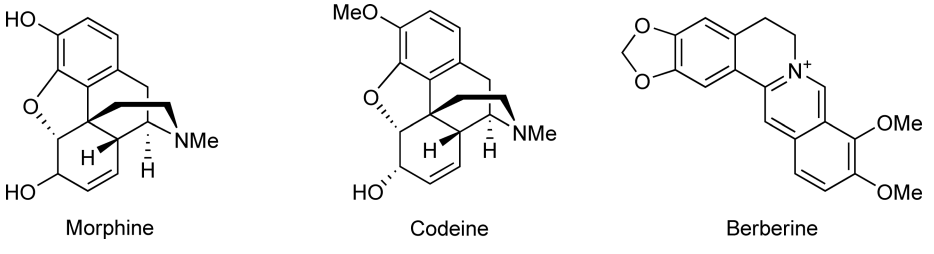


Figure 7 Representative tetrahydroisoquinoline alkaloids morphine, codeine, and berberine.

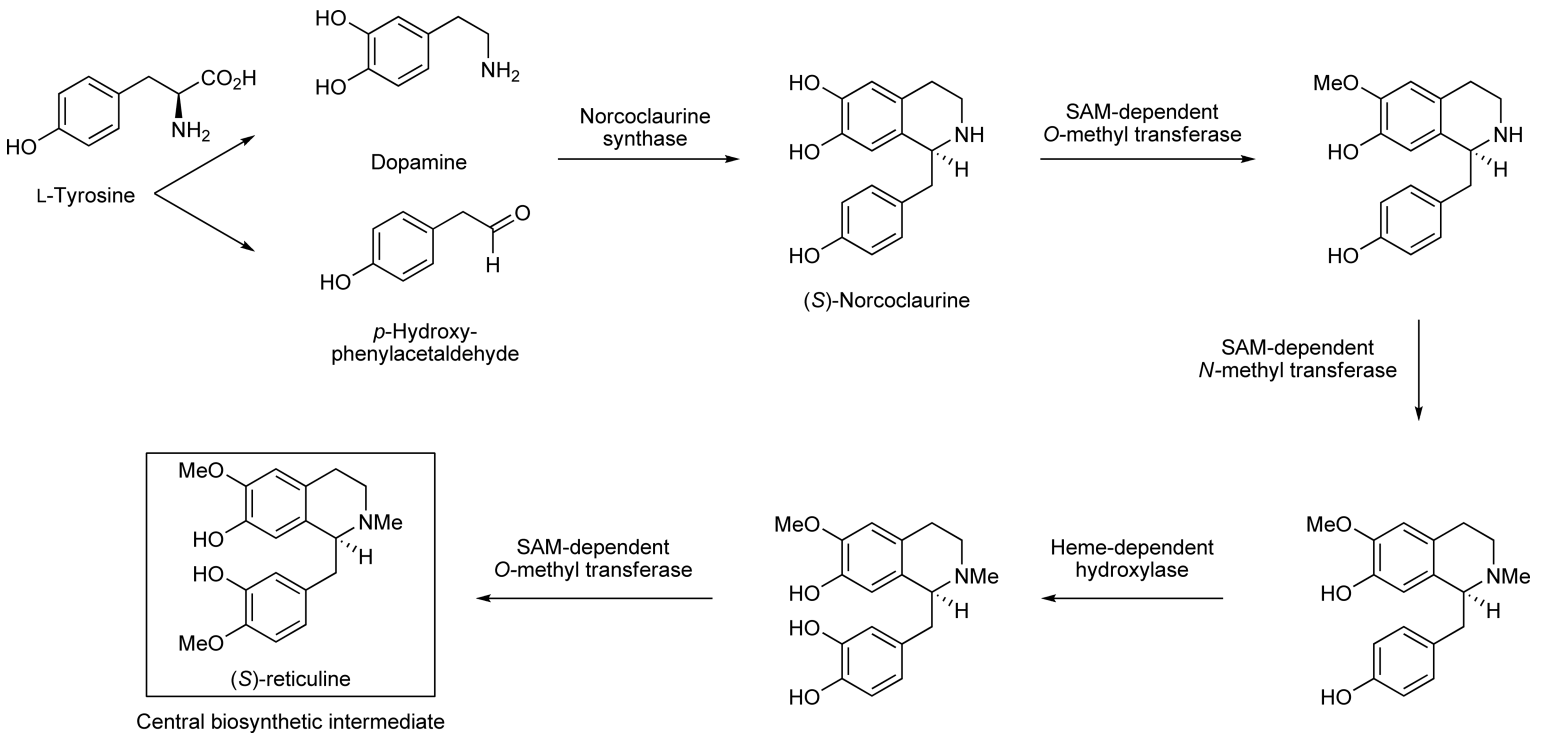


Figure 8 Biosynthesis of (*S*)-reticuline, the central intermediate of tetrahydroisoquinoline alkaloid biosynthesis.

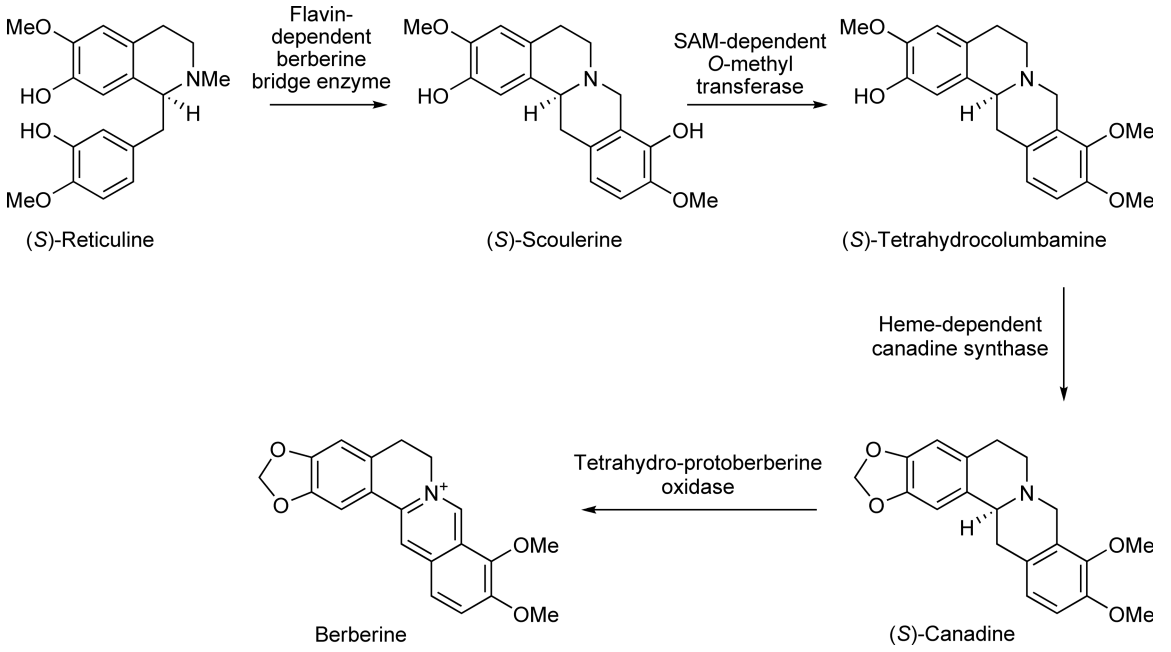


Figure 9 Biosynthesis of berberine.

biosynthesis is catalyzed by a substrate-specific oxidase, tetrahydroprotoberberine oxidase, the sequence of which has not been identified yet.[55] Berberine can be overproduced in *C. japonica* cell suspension cultures with reported productivity of berberine reaching 7 g l^{-1}.[56,57] This overproduction is one of the first demonstrations of production of a benzylisoquinoline alkaloid in cell culture at levels necessary for economic production. Additionally, this cell line has enabled the identification of many of the biosynthetic enzymes.

A second major pathway branch is the biosynthesis of the highly oxidized benzo(*c*)phenanthidine alkaloid sanguinarine, which is produced in a variety of plants and competes with morphine production in opium poppy. The pathway to sanguinarine has been elucidated at the enzymatic level (**Figure 10**).[58] Sanguinarine biosynthesis starts from (*S*)-scoulerine, as in berberine biosynthesis. Methylenedioxy bridge formation is then catalyzed by the P-450 cheilanthifoline synthase to yield cheilanthifoline.[59] A second P-450 enzyme, stylopine synthase, catalyzes the formation of the second methyenedioxy bridge of stylopine. Stylopine synthase from *E. californica* has been cloned recently.[60] Stylopine then is N-methylated by (*S*)-tetrahydroprotoberberine *cis-N*-methyltransferase to yield (*S*)-*cis-N*-methylstylopine, an enzyme that has been cloned recently from opium poppy.[61] A third P-450 enzyme, (*S*)-*cis-N*-methylstylopine hydroxylase, then forms protopine. Protopine is hydroxylated by a fourth P-450 enzyme, protopine 6-hydroxylase, to yield an intermediate that rearranges to dihydrosanguinarine.[62] The copper-dependent oxidase dihydrobenzophenanthridine oxidase, which has been purified,[63,64] then catalyzes the formation of sanguinarine from dihydrosanguinarine.

A third major branch leading to morphine biosynthesis has been investigated in *P. somniferum* cells and tissue. Notably, in morphine biosynthesis, (*S*)-reticuline is converted to (*R*)-reticuline, epimerizing the stereocenter generated by norcoclaurine synthase at the start of the pathway (**Figure 11**). This clearly exemplifies that the shortest enzymatic route to the final product is not always utilized by nature in metabolic pathways. Instead, nature will often use an existing biosynthetic intermediate for the biosynthesis. (*S*)-Reticuline is converted to (*R*)-reticuline through a 1,2-dehydroreticuline intermediate. Dehydroreticuline synthase catalyzes the oxidation of (*S*)-reticuline to 1,2-dehydroreticulinium ion.[65] This enzyme has not been cloned but has been purified partially and shown to be membrane associated. This intermediate then is reduced by dehydroreticuline reductase, an NADPH-dependent enzyme that stereoselectively transfers a hydride to dehydroreticulinium ion to yield (*R*)-reticuline. This enzyme has not been cloned yet but has been purified to homogeneity.[66] The key carbon–carbon bond of the morphinan alkaloids is formed by the cytochrome P-450 enzyme salutaridine

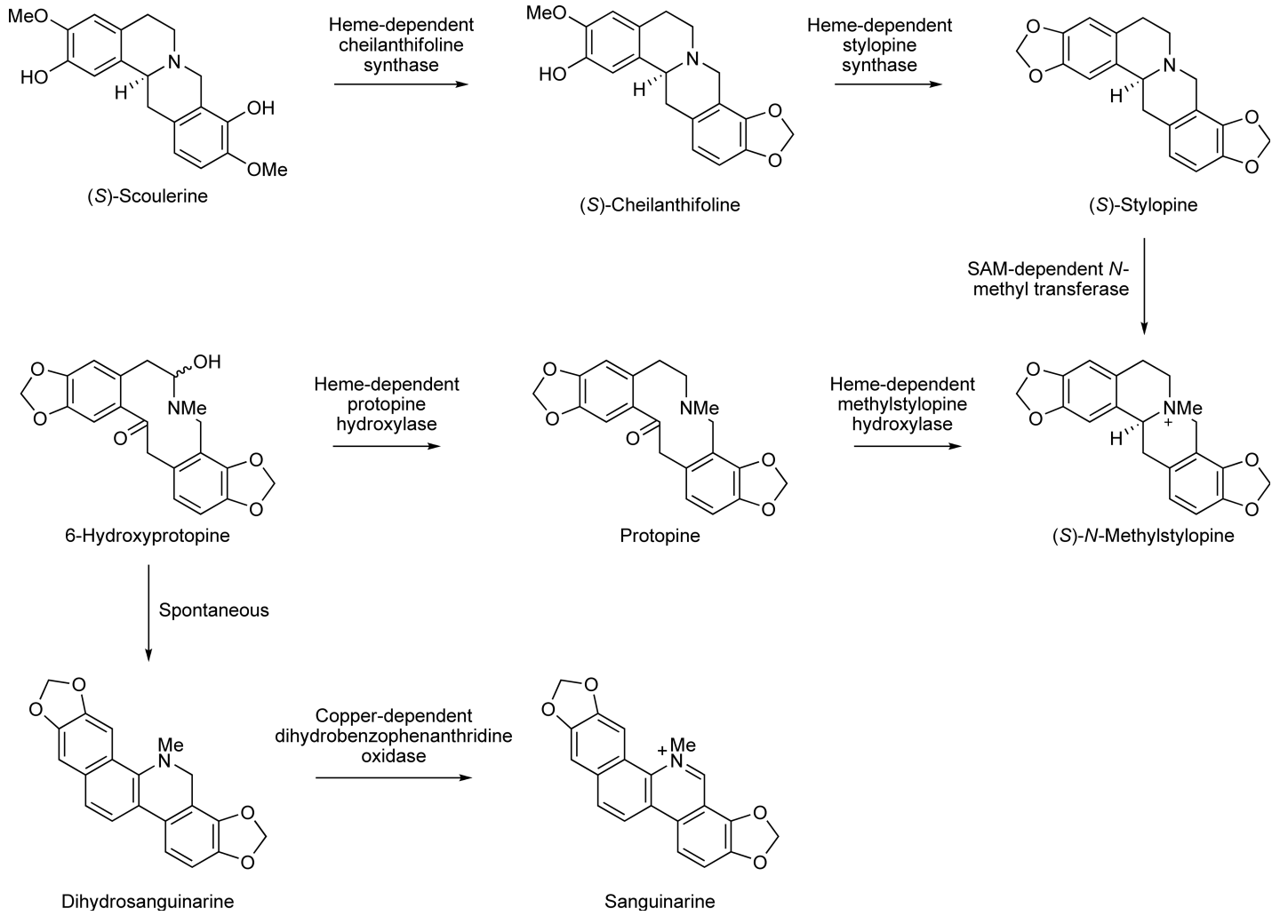


Figure 10 Biosynthesis of sanguinarine.

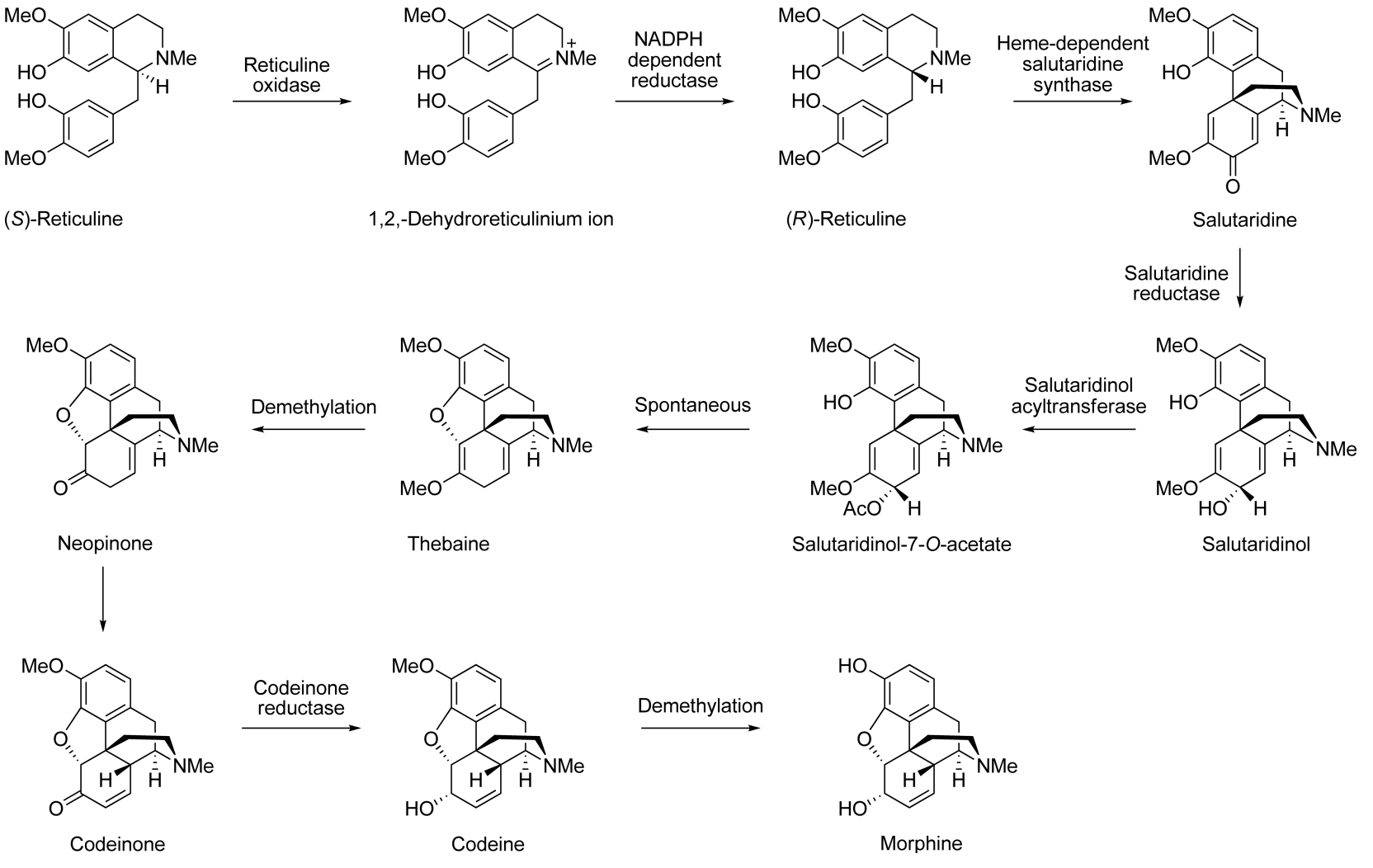


Figure 11 Biosynthesis of morphine and codeine.

synthase. Activity for this enzyme has been detected in microsomal preparations, and the sequence has been recently deposited.[67] The keto moiety of the resulting product, salutaridine, then is stereoselectively reduced by the NADPH-dependent salutaridine reductase to form salutardinol. Recent transcript analysis profile of *P. sominiferum* has resulted in the identification of this gene.[68] Salutaridinol acetyltransferase, also cloned, transfers an acyl group from acetyl-CoA to the newly formed hydroxyl group, which leads to the formation of salutaridinol-7-*O*-acetate.[69] The molecule can then undergo a spontaneous reaction in which the acetate acts as a leaving group. The resulting product, thebaine, then is demethylated by an as yet uncharacterized enzyme to yield neopinione, which exists in equilibrium with its tautomer codeinone. The NADPH-dependent codeinone reductase catalyzes the reduction of codeinone to codeine and has been cloned.[70,71] Finally, codeine is demethylated by an uncharacterized enzyme to yield morphine.

A series of elegant labeling experiments with human cell culture has indicated that mammalian tissue is capable of synthesizing morphine from dopamine precursors.[72] Presumably, morphine may play a role as an endogenous pain reliever in humans and other mammals. The mammalian enzymes involved in morphine biosynthesis have not been extensively investigated.

The localization of morphine biosynthesis has been investigated at the cellular level in intact poppy plants by using *in situ* RNA hybridization and immunofluorescence microscopy. The localization of 4′-*O*-methyltransferase (reticuline biosynthesis), berberine bridge enzyme (saguinarine biosynthesis), salutaridinol acetyltransferase (morphine biosynthesis), and codeinone reductase (morphine biosynthesis) has been probed. 4′-*O*-Methyltransferase and salutaridinol acetyltransferase are localized to parenchyma cells, whereas codeinone reductase is localized to laticifer cells in sections of capsule (fruit) and stem from poppy plants. Berberine bridge enzyme is found in parenchyma cells in roots. Therefore, this study suggests that two cell types are involved in isoquinoline biosynthesis in poppy and that intercellular transport is required for isoquinoline alkaloid biosynthesis.[73] Another study, however, implicates a single cell type (sieve elements and their companion cells) in isoquinoline alkaloid biosynthesis.[74,75] Therefore, it is not clear whether transport of pathway intermediates is required for alkaloid biosynthesis or whether the entire pathway can be performed in one cell type. Undoubtedly, future studies will provide more insight into the trafficking involved in plant secondary metabolism.

The extensive knowledge of the genes of isoquinoline biosynthesis has enabled a variety of metabolic engineering work to be done. In attempts to accumulate thebaine and decrease production of morphine (a precursor to heroin), codeinone reductase in opium poppy plant was downregulated by using RNAi.[35,76] Silencing of codeinone reductase results in the accumulation of (*S*)-reticuline but not the substrate codeinone or other compounds on the pathway from (*S*)-reticuline to codeine (**Figure 11**). The cytochrome P-450 responsible for the oxidation of (*S*)-*N*-methylcoclaurine to (*S*)-3′-hydroxy-*N*-methylcocluarine has been overexpressed in opium poppy plants, and morphinan alkaloid production in the latex is increased subsequently to 4.5 times the level in wild-type plants.[77] Additionally, suppression of this enzyme resulted in a decrease in morphinan alkaloids to 16% of the wild-type level. Notably, analysis of a variety of biosynthetic gene transcript levels in these experiments supports the hypothesis that this P-450 enzyme plays a regulatory role in the biosynthesis of benzylisoquinoline alkaloids. Collectively, these studies highlight that the complex metabolic networks found in plants are not redirected easily or predictably in all cases. Notably, portions of this pathway have been reconstituted in *Saccharomyces cerevisiae*, which is an organism that is much more amenable to rational metabolic engineering efforts.[78,79]

1.25.6 Monoterpene Indole Alkaloid Biosynthesis

The terpene indole alkaloids are a diverse class of natural products, comprising over 2000 members. These complex natural products possess a range of chemical structures and a wealth of biological activities (**Figure 12**).[80,81] The biosynthetic pathways of some classes of terpene indole alkaloids are well understood, and in some branches, many of the enzymes that are responsible for biosynthesis have been actually cloned and mechanistically studied *in vitro*. In other cases, the biosynthetic pathway is only proposed based on the results of feeding studies with isotopically labeled substrates and from the structures of isolated biosynthetic intermediates. Although many biosynthetic genes from this pathway remain unidentified, recent studies have correlated

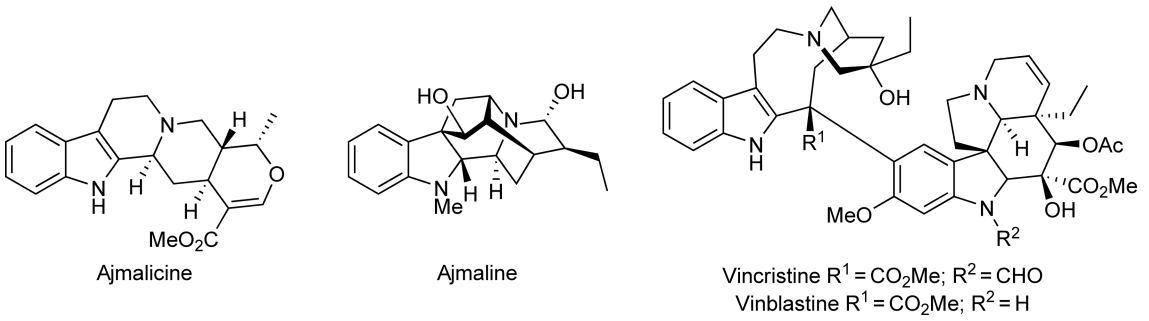


Figure 12 Representative monoterpene indole alkaloids ajmalicine, ajmaline, vinblastine, and vincristine.

terpenoid indole alkaloid production with the transcript profiles of *Catharanthus roseus* cell cultures.[82] Although the genome sequences of none of these alkaloid-producing plants is available, a number of expressed sequence tag (EST) libraries for *C. roseus* have been reported.[83,84]

All terpenoid indole alkaloids are derived from tryptophan and the iridoid terpene secologanin (**Figure 13**). The involvement of an iridoid monoterpene in these indole alkaloid pathways was first proposed after the structures of several iridoid terpenes were elucidated, and secologanin was identified as the specific iridoid precursor.[85–87] Secologanin is itself a natural product, and the biosynthetic pathway for this molecule has not been fully elucidated, although feeding studies with *C. roseus* cell suspension cultures and ^{13}C-glucose strongly suggest that secologanin is ultimately derived from the triose phosphate/pyruvate or 'nonmevalonate'

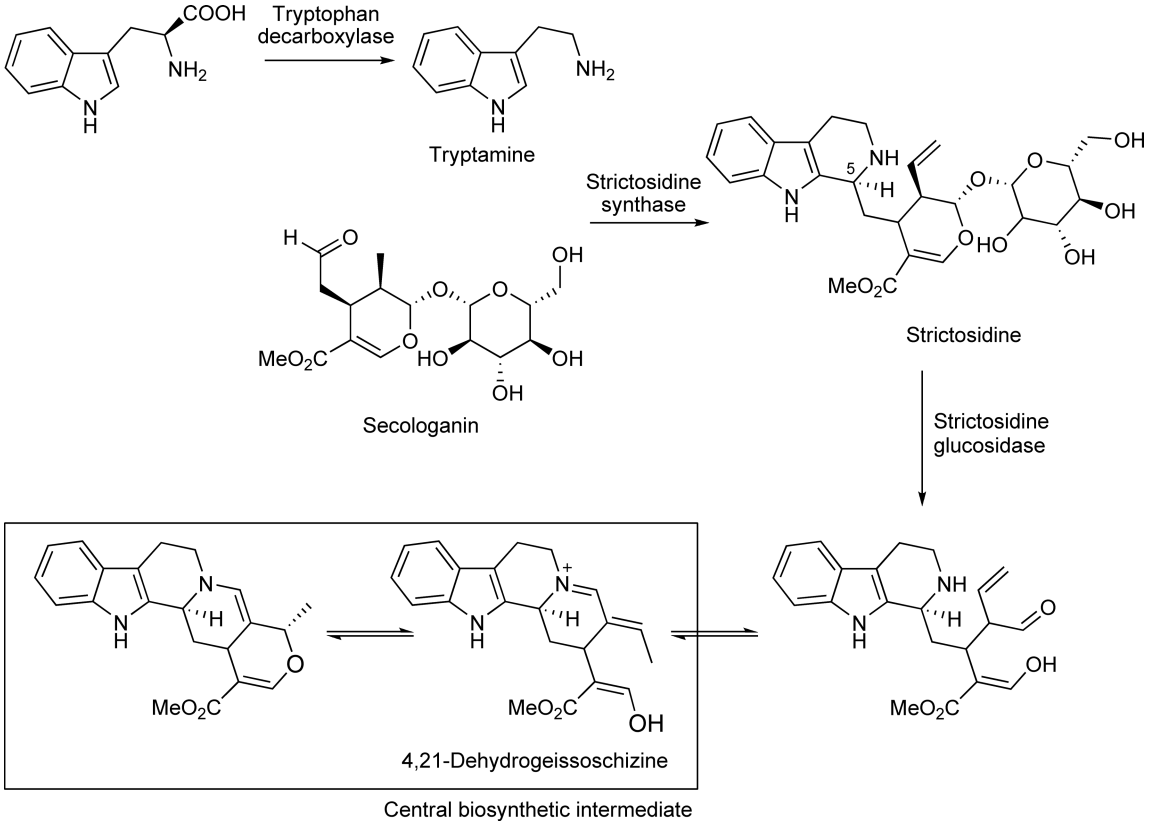


Figure 13 Biosynthesis of deglycosylated strictosidine, the central biosynthetic intermediate in monoterpene indole alkaloid biosynthesis.

pathway.[88] Several enzymes involved in the biosynthesis of Isoprenylpyrophosphate-1-deoxy-D-xylulose 5-phosphate (IPP)–2-Methyl-D-erythritol-4-phosphate (DXP) synthase, DXP reductoisomerase, and MEP synthase – have been cloned from *C. roseus*.[89,90] Several other genes involved in the later steps of secologanin biosynthesis, namely geraniol-10-hydroxylase,[91,92] and secologanin synthase[93,94] have been identified. Tryptophan decarboxylase, a pyridoxal-dependent enzyme, converts tryptophan to tryptamine to yield the amine-containing starting material for this family of alkaloids.[95,96] The enzyme strictosidine synthase catalyzes a stereoselective Pictet–Spengler condensation between tryptamine and secologanin to yield strictosidine. Strictosidine synthase[97] has been cloned from the plants *C. roseus*,[98] *Rauwolfia serpentina*,[99] and *Ophiorrhiza pumila*.[100] A crystal structure of strictosidine synthase from *R. serpentina* has been reported,[101] and a number of reports have indicated that the substrate specificity of the enzyme can be modulated.[102–104]

In most monoterpene indole alkaloids, strictosidine is deglycosylated by a dedicated β-glucosidase, which converts the substrate to a reactive hemiacetal intermediate.[105–107] This hemiacetal opens to form a dialdehyde intermediate, which then forms dehydrogeissoschizine. The enol form of dehydrogeissoschizine can undergo 1,4 conjugate addition to produce the heteroyohimbine cathenamine, as well as a variety of other isomers.[107–111]

The biosynthetic pathway for ajmaline in *R. serpentina* is one of the best-characterized branches of the terpenoid indole alkaloid pathways (**Figure 14**). Much of this progress has been detailed in an extensive review.[112] Like all other terpenoid indole alkaloids, ajmaline, an antiarrhythmic drug with potent sodium channel-blocking properties,[113] is derived from deglycosylated strictosidine. At least eight enzymes are predicted to catalyze the subsequent steps of ajmaline biosynthesis that occur after strictosidine deglycosylation. The sarpagan alkaloid, polyneuridine aldehyde, is a known early intermediate of the ajmaline pathway. A mechanism in which the sarpagan bridge enzyme transforms an isomer of deglycosylated strictosidine to polyneuridine aldehyde has been proposed.[114] A membrane-protein fraction of an *R. serpentina* extract transformed labeled strictosidine into sarpagan-type alkaloids. The enzyme activity was shown to be dependent on NADPH and molecular oxygen, suggesting that sarpagan bridge enzyme may be a cytochrome P-450

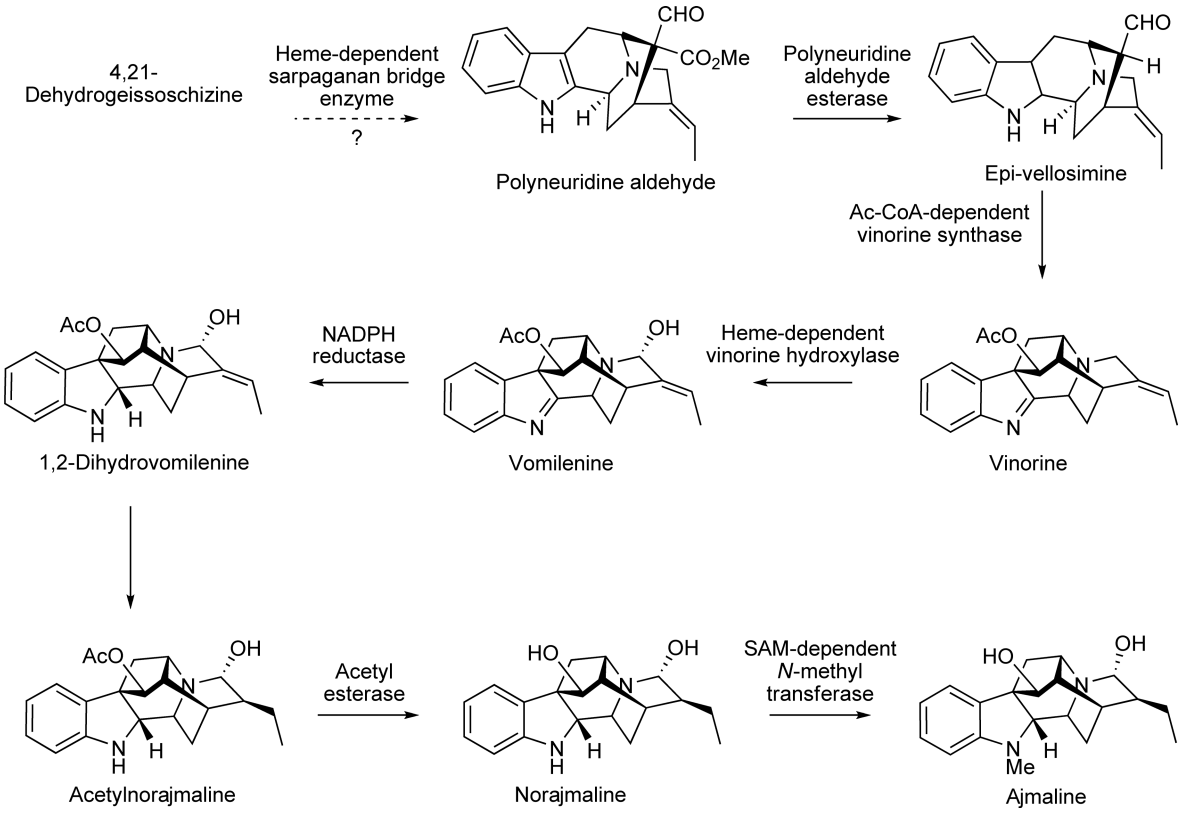


Figure 14 Biosynthesis of ajmaline.

enzyme.[115,116] Isolation of this enzyme will yield further insight into this key step that commits the deglycosylated strictosidine intermediate to the sarpagan- and ajmalan-type alkaloid pathways.

Polyneuridine aldehyde esterase then hydrolyzes the polyneuridine aldehyde methyl ester, which generates an acid that spontaneously decarboxylates to yield epi-vellosamine. This enzyme has been cloned from a *Rauwolfia* cDNA library, heterologously expressed in *E. coli,* and subjected to detailed mechanistic studies.[117–119] Polyneuridine aldehyde esterase appears to be a member of the α/β hydrolase super family and contains a Ser, His, Asp catalytic triad.[117–119] Site-directed mutagenesis indicates that each residue of the catalytic triad is required for activity. Vinorine synthase transforms the sarpagan alkaloid epi-vellosamine to the ajmalan alkaloid vinorine.[120] Vinorine synthase also has been purified from *Rauwolfia* cell culture, subjected to protein sequencing, and cloned from a cDNA library.[121,122] The enzyme, which seems to be an acetyltransferase homolog, has been crystallized and subjected to site-directed mutagenesis studies of this protein, leading to a proposed mechanism.[123] Vinorine hydroxylase hydroxylates vinorine to form vomilene.[124] Vinorine hydroxylase seems to be a P-450 enzyme, and has not been cloned yet. The indolenine bond of vomilene is reduced by an NADPH-dependent reductase to yield 1,2-dihydrovomilenene. A second enzyme, 1,2-dihydrovomilenene reductase, then reduces this product to acetylnorajmaline. Partial protein sequences have been obtained for both of the purified reductases. Although several putative clones that encode these proteins have been isolated, the activity of these clones has not been verified yet.[125,126] An acetylesterase then hydrolyzes the acetyl link of acetylnorajmaline to yield norajmaline. This esterase has been purified from *R. serpentina* cell suspension cultures, and a full-length clone has been isolated from a cDNA library. Expression of the gene in tobacco leaves successfully yielded protein with the expected enzymatic activity.[127] In the final step of ajmaline biosynthesis, an *N*-methyltransferase introduces a methyl group at the indole nitrogen of norajmaline. Although this enzymatic activity has been detected in crude cell extracts, the enzyme has not been characterized additionally.[128] In summary, the enzymatic activities for all steps of ajmaline biosynthesis have been detected. Strictosidine synthase, strictosidine glucosidases, polyneuridine aldehyde esterase, vinorine synthase, and 17-*O*-acetyl-ajmalanesterase have been cloned. Putative clones for vinorine hydroxylase, vomilenine reductase, and 1,2-dihydrovomilenen reductase have been isolated. *N*-Methyltransferase activity and sarpagan bridge enzyme activities have only been detected in crude cell extracts.

Ajmalicine (raubasine) affects smooth muscle function and is used to help prevent strokes,[129] and tetrahydroalstonine exhibits antipsychotic properties (**Figure 15**).[130] These compounds are found in a variety of plants, including *C. roseus* and *R. serpentina.* A partially purified NADPH-dependent reductase isolated from a tetrahydroalstonine that produces a *C. roseus* cell line was shown to catalyze the conversion of cathenamine, a spontaneous reaction product that results after strictosidine deglycosylation, to tetrahydroalstonine *in vitro* (**Figure 15**).[131] A second *C. roseus* cell line contains an additional reductase that produces ajmalicine. Labeling studies performed with crude *C. roseus* cell extracts in the presence of D_2O or deuterated, reduced form of Nicotinamide adenine dinucleotide phosphate (NADPD) support a mechanism in which the reductase acts on the iminium form of cathenamine.[132]

Vindoline, an aspidosperma-type alkaloid produced by *C. roseus*, is a key precursor for vinblastine, an anticancer drug that is the most important pharmaceutical product of *C. roseus* (**Figure 16**). Vindoline, like ajmalicine and ajmaline, is produced from deglycosylated strictosidine. Deglycosylated strictosidine is converted to tabersonine, an aspidosperma-type alkaloid, through a series of biochemical steps for which no enzymatic information exists. Studies by numerous groups in the 1960s and 1970s enabled detailed hypothetical proposals of the biosynthesis of aspidosperma-type alkaloids in *C. roseus.*[133–143] These proposed pathways are based on feeding studies of isotopically labeled substrates to seedlings or shoots, isolation of discrete intermediates from plant materials, and from biomimetic model reactions. More details are known about the elaboration of tabersonine to vindoline.[144] Tabersonine-16-hydroxylase, a cytochrome P-450, hydroxylates tabersonine to 16-hydroxy-tabsersonine.[145,146] This hydroxyl group is then methylated by a SAM-dependent *O*-methyltransferase to yield 16-methoxy-tabersonine; this enzyme (16-hydroxytabersonine-16-*O*-methyltransferase) has recently been cloned.[147] In the next step, hydration of a double bond by an uncharacterized enzyme produces 16-methoxy-2,3-dihydro-3-hydroxytabersonine. Transfer of a methyl group to the indole nitrogen by an *N*-methyltransferase yields desacetoxyvindoline. This methyltransferase activity has been detected only in differentiated plants, not in plant cell cultures.[148] The resulting intermediate, deacetylvindoline, is produced by the oxoglutarate-dependent dioxygenase enzyme desacetylvindoline 4-hydroxylase. This enzyme has been cloned and is also absent from plant cell cultures.[149] In the last step,

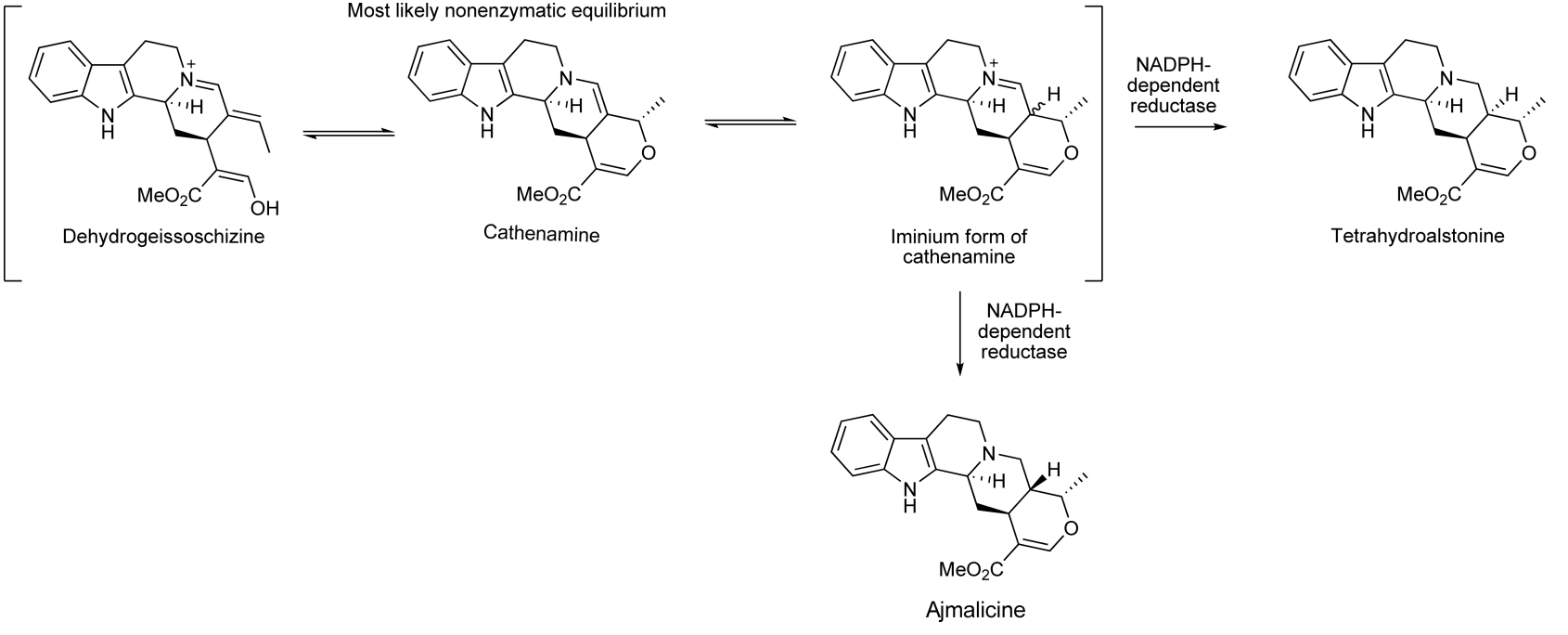


Figure 15 Biosynthesis of ajmalicine and tetrahydroalstonine.

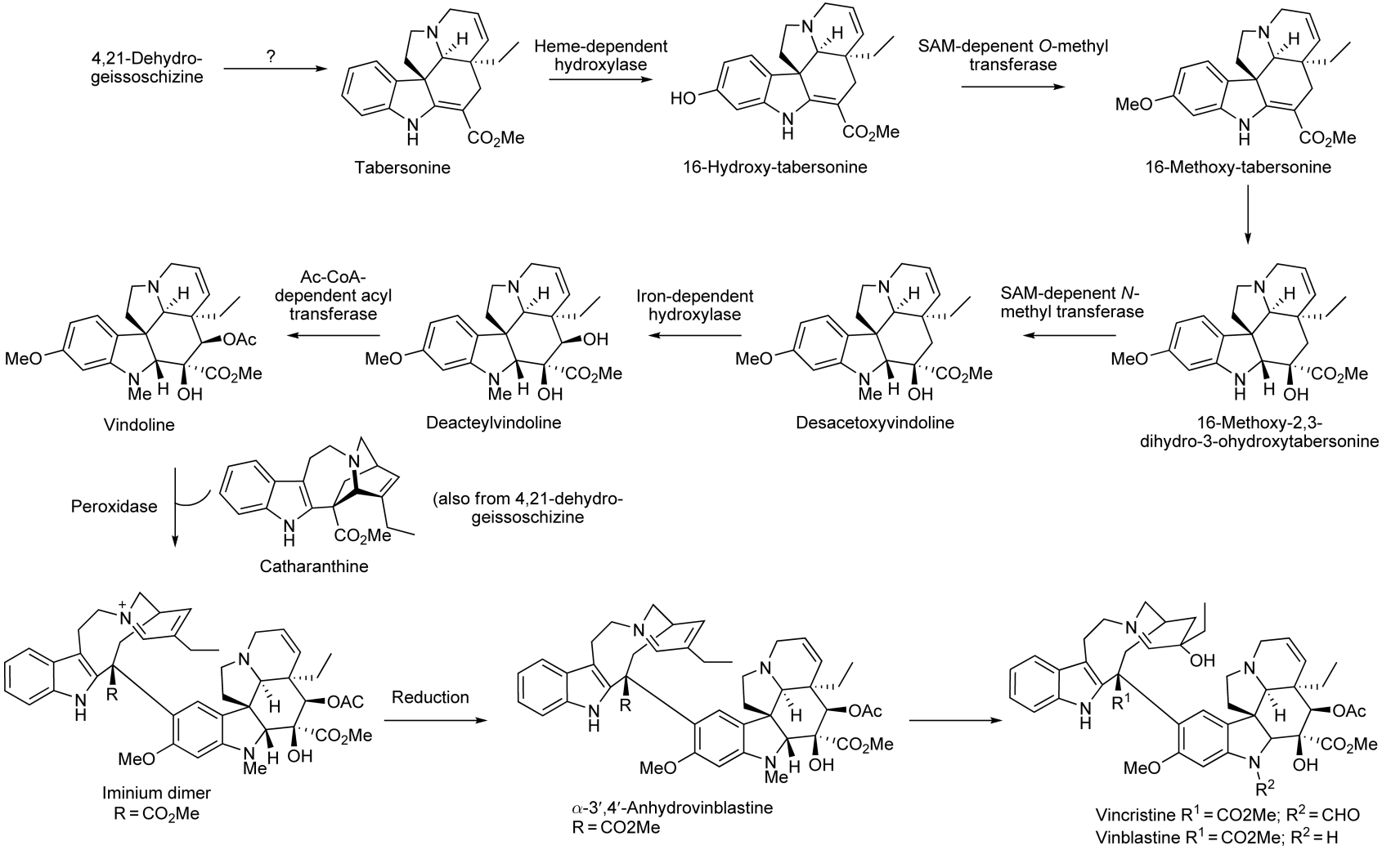


Figure 16 Biosynthesis of vindoline and vinblastine.

desacetylvindoline is acetylated by desacetylvindoline *O*-acetyltransferase. This enzyme, also absent from nondifferentiated plant material, has been cloned successfully.[150]

Vinblastine and the structurally related vincristine are highly effective anticancer agents currently used clinically against leukemia and other cancers.[151,152] Inspection of these bisindole alkaloids indicates that they are derived from coupling of vindoline and catharanthine, which is believed to proceed via the formation of an iminum intermediate with catharanthine (**Figure 16**). This iminium intermediate is reduced to form anhydrovinblastine, a naturally occurring compound in *C. roseus* plants.[153]

Peroxidase-containing fractions of plant extracts were found to catalyze the formation of the bisindole dehydrovinblastine from catharanthine and vindoline.[154,155] The peroxidase CRPRX1 (α-3′,4′-anhydrovinblastine synthase), purified and cloned from *C. roseus* leaves, has been demonstrated to convert vindoline and catharanthine to anhydrovinblastine.[156,157] Catharanthine is most likely oxidized to an iminium ion, which then reacts with the relatively nucleophilic vindoline.[158] Although this peroxidase is not highly substrate specific for catharanthine and vindoline, localization studies strongly suggest that CRPRX1 is the dedicated peroxidase for bisindole formation.[157,158] Finally, after formation of dehydrovinblastine, hydroxylation of the double bond yields vinblastine, and oxidation of the *N*-methyl group yields vincristine.

As in morphine biosynthesis, the knowledge of the enzyme sequences allows a more detailed understanding of the localization of the enzymes.[159] Strictosidine synthase seems to be localized to the vacuole,[98] and strictosidine glucosidase is believed to be associated with the cytosol and the membrane of the endoplasmic reticulum.[160] Tabersonine-16-hydroxylase is associated with the endoplasmic reticulum membrane;[145] *N*-Methyltransferase activity is believed to be associated with the thylakoid, a structure located within the chloroplast;[148,161] and vindoline-4-hydroxylase and desacetylvindoline *O*-acetyltransferase are believed to be localized to the cytosol.[161,162] In addition to subcellular compartmentalization, specific cell types are required for the biosynthesis of some terpenoid alkaloids, as is the case in morphine biosynthesis described above. Several enzymes involved in the early stages of secologanin biosynthesis seem to be localized to the phloem parenchyma, as evidenced by immunocytochemistry and *in situ* RNA hybridization studies.[163] However, additional studies have suggested that these genes also are observed in the epidermis and laticifers.[164] Studies of the localization of vindoline biosynthetic enzymes by using immunocytochemistry and *in situ* RNA hybridization strongly suggest that the midpart of the vindoline pathway (tryptophan decarboxylase, strictosidine synthase, and tabersonine-16-hydroxylase) takes place in epidermal cells of leaves and stems. However, the later steps catalyzed by desacetylvindoline 4-hydroxylase and desacetylvindoline *O*-acetyltransferase take place in specialized cells, the laticifers, and idioblasts.[165] As with isoquinoline alkaloid biosynthesis, deconvolution of the enzyme localization patterns remains a challenging endeavor.

Again, the partially elucidated pathways of monoterpene indole alkaloid biosynthesis have allowed metabolic engineering efforts. These studies have primarily taken place in *C. roseus*. Strictosidine synthase and tryptophan decarboxylase have been overexpressed in *C. roseus* cell cultures.[166,167] Generally, overexpression of tryptophan decarboxylase does not seem to have a significant impact on alkaloid production, although overexpression of strictosidine synthase does seem to improve alkaloid yields. Overexpression of tryptophan and secologanin biosynthetic enzymes in *C. roseus* hairy root cultures resulted in modest increases in terpenoid indole alkaloid production.[168,169] Secologanin biosynthesis seems to be the rate-limiting factor in alkaloid production.[170] Using a combination of unnatural tryptamine analogs and a reengineered strictosidine synthase enzyme, the biosynthetic pathway can be used to produce alkaloid derivatives.[171,172] Although strictosidine synthase and strictosidine glucosidase enzymes have been expressed heterologously in yeast,[173] efforts to express heterologously terpenoid indole alkaloids currently are limited because the majority of the biosynthetic genes remain uncloned.

Transcription factors that upregulate strictosidine synthase,[174] as well as a transcription factor that coordinately upregulates expression of several terpenoid indole alkaloid biosynthetic genes, have been found.[14] Several zinc finger proteins that act as transcriptional repressors to tryptophan decarboxylase and strictosidine synthase also have been identified.[175] Manipulation of these transcription factors may allow tight control of the regulation of terpenoid indole alkaloid production. Interestingly, expression of a transcription factor from *Arabidopsis thaliana* in *C. roseus* cell cultures results in an increase in alkaloid production.[176] The dramatic increases in alkaloid production that have been noted in morphine metabolic engineering efforts have for the most part not been observed in the monoterpene indole alkaloids. Notably, more enzymes in the isoquinoline

biosynthetic pathway are known. Presumably, as more monoterpene indole alkaloid biosynthetic enzymes are identified, one may be identified that has a significant impact on the alkaloid expression levels.

1.25.7 Tropane Alkaloid Biosynthesis

The tropane alkaloids hyoscyamine and scopolamine (**Figure 17**) function as acetylcholine receptor antagonists and are used clinically as parasympatholytics. The illegal drug cocaine is also a tropane alkaloid. The tropane alkaloids are biosynthesized primarily in plants of the family Solonaceae, which includes *Hyoscyamus*, *Duboisia*, *Atropa*, and *Scopolia*.[17,18] Nicotine, although perhaps not apparent immediately from its structure, is related biosynthetically to the tropane alkaloids.

Tropane alkaloid biosynthesis has been studied at the biochemical level, and several enzymes from the biosynthetic pathway have been isolated and cloned, although the pathway has not been elucidated completely at the genetic level (**Figure 18**).[177] In plants, L-arginine is converted to the nonproteogenic amino acid L-ornithine by the urease enzyme arginase. Ornithine decarboxylase then decarboxylates ornithine to yield the diamine putrescine. In *Hyoscyamus*, *Duboisia*, and *Atropa*, putrescine (so-named because of its odor) serves as the common precursor for the tropane alkaloids. Putrescine is N-methylated by a SAM-dependent methyltransferase that has been cloned to yield *N*-methylputrescine.[178,179] Putrescine *N*-methyltransferase now has been cloned from a variety of plant species,[180–182] and site-directed mutagenesis and homology models have led to insights into the structure–function relationships of this enzyme.[182] *N*-Methylputrescine is then oxidized by a diamine oxidase to form 4-methylaminobutanal, which then cyclizes, most likely nonenzymatically, to form the *N*-methyl-D-pyrrolinium ion.[183–185] This enzyme, which recently has been cloned, seems to be a copper-dependent amine oxidase.[186] Immunoprecipitation experiments suggest that this enzyme associates with the enzyme *S*-adenosylhomocysteine hydrolase.[187] The pyrrolinium ion is then converted to the tropanone skeleton by as yet uncharacterized enzymes. Although no enzymatic information is available, chemical labeling studies have indicated that an acetate-derived moiety condenses with the pyrrolinium ion.[17] Tropanone is then reduced via an NADPH-dependent reductase to tropine that has been cloned from *Hyoscyamus niger*.[188,189] All tropane-producing plants seem to contain two tropinone reductases, which create a branch point in the pathway. Tropinone reductase I yields the tropane skeleton, whereas tropinone reductase II yields the opposite stereocenter, pseudotropine.[190] Tropane is converted to scopolamine or hyoscyamine, while the tropinone reductase II product pseudotropine leads to calystegines.[191] These two tropinone reductases have been crystallized, and site-directed mutagenesis studies indicate that the stereoselectivity of the enzymes can be switched by rational protein engineering.[192,193]

The biosynthesis of scopolamine is the best characterized of the tropane alkaloids. After action by tropinone reductase I, tropine is condensed with phenylacetate through the action of a P-450 enzyme to form littorine.[13] The phenyllactate moiety is believed to derive from an intermediate involved in phenylalanine metabolism.[17] Littorine then undergoes rearrangement to form hyoscyamine. The enzyme that catalyzes this rearrangement was originally believed to proceed via a radical mechanism using SAM as the source of an adenosyl radical.[194–197] However, a large-scale RNAi study performed in *H. niger* suggests that a P-450 enzyme, followed by action of a reductase enzyme, is responsible for the rearrangement. Hyoscyamine 6β-hydroxylase

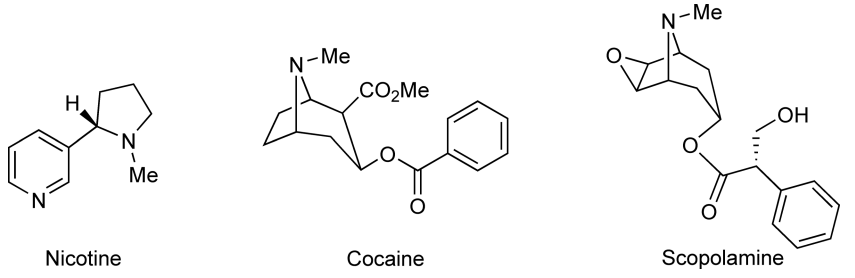


Figure 17 Representative type tropane and nicotinic alkaloids nicotine, cocaine, and scopolamine.

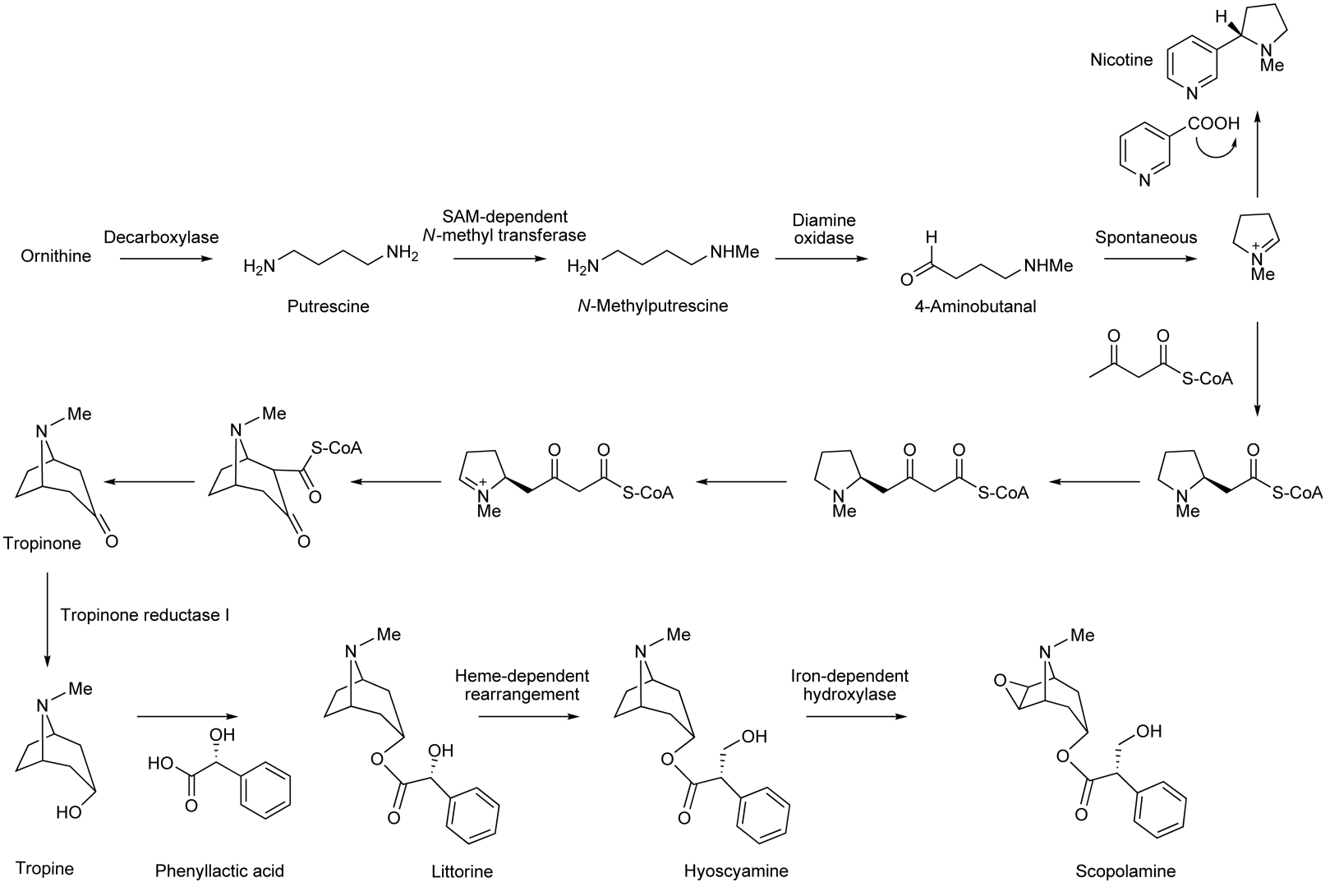


Figure 18 Biosynthesis of scopolamine.

catalyzes the hydroxylation of hyoscyamine to 6β-hydroxyhyoscyamine as well as the epoxidation to scopolamine.[198,199] Hyoscyamine 6β-hydroxylase, which has been cloned and expressed heterologously,[200] is a nonheme, iron-dependent, oxoglutarate-dependent protein. The epoxidation reaction appears to occur more slowly than the hydroxylation reaction. The tropane alkaloids seem to be formed in the roots and then transported to the aerial parts of the plant.[201]

Atropa belladonna plants have been transformed with hyoscyamine 6β-hydroxylase from *H. niger*. *A. belladonna* normally produces high levels of hyoscyamine, the precursor for the more pharmaceutically valuable alkaloid scopolamine. However, in one of the earliest demonstrations of plant natural product metabolic engineering, after transformation with hyoscyamine 6β-hydroxylase, transgenic *A. belladonna* plants were shown to accumulate scopolamine almost exclusively.[202] Additionally, the levels of tropane alkaloid production in a variety of hairy root cultures were altered by overexpression of methyltransferase putrescine-*N*-methyltransferase and hyoscyamine 6β-hydroxylase. Overexpression of both of these enzymes in a hairy root cell culture resulted in significant increases in scopolamine production.[202,203] Fluorinated phenyllactic acid substrates could be incorporated into the pathway,[204] and several substrates derived from putrescine analogs were turned over by the enzymes of several Solonaceae species.[205]

1.25.8 Purine Alkaloid Biosynthesis

Caffeine, a purine alkaloid, is one of the most widely ingested of all natural products. Caffeine is a natural component of coffee, tea, and cocoa, and the impact of caffeine on human health has been studied extensively. The biosynthetic pathway of caffeine has been elucidated recently on the genetic level (**Figure 19**), and most work has focused on the plant species *Coffea* (coffee) and *Camellia* (tea).[19,206] Xanthosine, which is derived from purine metabolites, is the first committed intermediate in caffeine biosynthesis. Xanthosine can be formed from *de novo* purine biosynthesis, SAM cofactor, the adenylate pool, and the guanylate pool.[19] *De novo* purine biosynthesis and the adenosine from SAM are believed to be the most important sources of xanthosine.[19,207] Xanthosine is methylated to yield *N*-methylxanthosine by the enzyme xanthosine *N*-methyltransferase (XMT) (also called 7-methylxanthosine synthase).[208–210] *N*-Methylxanthosine is converted to *N*-methylxanthine by methylxanthine nucleosidase, an enzyme that has not been cloned, but is present in many noncaffeine-producing organisms.[211] *N*-Methylxanthine is converted to theobromine by 7-methylxanthine-*N*-methyltransferase (MXMT) (also called theobromine synthase), a second *N*-methyltransferase.[209,212] Theobromine is converted to caffeine by a final *N*-methyltransferase, dimethylxanthine-*N*-methyltransferase (DXMT) (also called caffeine synthase).[209]

Coffee and tea plants seem to contain a variety of *N*-methyltransferase enzymes that have varying substrate specificity.[19,206] For example, a caffeine synthase enzyme isolated from tea leaves catalyzes both the N-methylation of *N*-methylxanthine and theobromine.[213] The substrate specificity of the methyltransferases can be changed by site-directed mutagenesis.[214]

Coffee beans with low caffeine levels could be valuable commercially, given the demand for decaffeinated coffee. Because of the discovery of these *N*-methyltransferase genes, genetically engineered coffee plants with reduced caffeine content now can be constructed.[212,215] For example, a 70% reduction in caffeine content in *Coffea* was obtained by downregulating MXMT (theobromine synthase) using RNAi.[216] Additionally, the promoter of one of the *N*-methyltransferases has been discovered recently, which may allow transcriptional gene silencing.[217]

1.25.9 Conclusions and Outlook

Alkaloids constitute a structurally diverse array of natural products, and these compounds have a wide range of biological activities. Many have important pharmaceutical uses. Plants are regarded as the oldest source of alkaloids, and some of the most widely recognized alkaloids, such as morphine, quinine, strychnine, and cocaine, are derived from plants. However, rapid advances in molecular biology and sequencing of bacterial and fungal genomes have fostered the discovery of new alkaloids in these simpler microbial organisms, and a

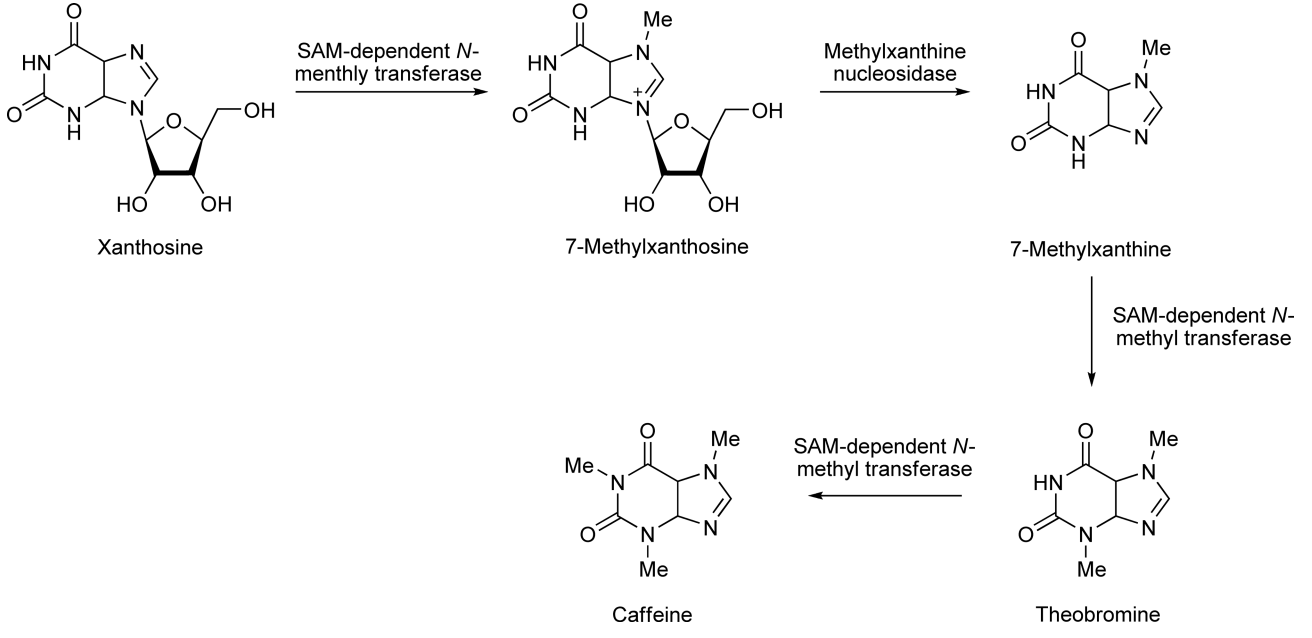


Figure 19 Biosynthesis of caffeine.

wealth of biosynthetic information for these compounds has been obtained in a number of recent mechanistic studies. Undoubtedly, many more microbe-derived alkaloids remain to be discovered. Given the medicinal importance of many alkaloids, metabolic engineering efforts serve as an important application of biosynthetic studies. Metabolic engineering has been used to increase the production levels of alkaloids, reconstitute alkaloid biosynthesis in simpler host organisms, and rationally modify the structure of alkaloids by engineering of the biosynthetic enzymes. The past several years have resulted in major advances in alkaloid discovery, biosynthetic pathway elucidation, and metabolic engineering.

Glossary

acridine-type alkaloids Anthranilic acid-derived natural products.
alkaloid Nitrogen-containing organic substances of natural origin with basic character.
indolizidine alkaloids Lysine-derived natural products.
monoterpene indole alkaloids Tryptophan- and secologanin-derived natural products.
piperdine alkaloids Lysine-derived natural products.
pyrrolizidine alkaloids Ornithine-derived natural products.
quinazoline alkaloids Anthranilic acid-derived natural products.
quinolizidine alkaloids Lysine-derived natural products.
tetrahydroisoquinoline alkaloids Tyrosine-derived natural products.
tropane alkaloids Ornithine-derived natural products.

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Biographical Sketch

Sarah E. O'Connor received a bachelor of science degree in chemistry from the University of Chicago. She received her Ph.D. in chemistry under the direction of Barbara Imperiali at Caltech and MIT, where her thesis work focused on the synthesis and structural analysis of N-linked glycopeptides. She did her postdoctoral research with Chris Walsh at Harvard Medical School where she studied the biosynthesis of epothilone and several other polyketide and peptide natural products. She began her independent research program at MIT where her group is investigating the biosynthesis of plant-derived alkaloid natural products.