9

ALKALOIDS

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How nature synthesizes complex secondary metabolites, or natural products, can be studied only by working within the disciplines of both chemistry and biology. Alkaloids are a complex group of natural products with diverse mechanisms of biosynthesis. This chapter highlights the biosynthesis of four major classes of plant-derived alkaloids. Only plant alkaloids for which significant genetic information has been obtained were chosen for review. Isoquinoline alkaloid, terpenoid indole alkaloid, tropane alkaloid, and purine alkaloid biosynthesis are described here. The chapter is intended to provide an overview of the basic mechanism of biosynthesis for selected members of each pathway. Manipulation of these pathways by metabolic engineering is highlighted also.

Alkaloids are a highly diverse group of natural products related only by the presence of a basic nitrogen atom located at some position in the molecule. Even among biosynthetically related classes of alkaloids, the chemical structures are often highly divergent. Although some classes of natural products have a recognizable biochemical paradigm that is centrally applied throughout the pathway, for example, the "assembly line" logic of polyketide biosynthesis (1), the biosynthetic pathways of alkaloids are as diverse as the structures. It is difficult to predict the biochemistry of a given alkaloid based solely on precedent, which makes alkaloid biosynthesis a challenging, but rewarding, area of study.

Natural Products in Chemical Biology, First Edition. Edited by Natanya Civjan.

9.1 BIOLOGIC BACKGROUND

Hundreds of alkaloid biosynthetic pathways have been studied by chemical strategies, such as isotopic labeling experiments (2, 3). However, modern molecular biology and genetic methodologies have facilitated the identification of alkaloid biosynthetic enzymes. This chapter focuses on pathways for which a significant amount of genetic and enzymatic information has been obtained. Although alkaloid natural products are produced by insects, plants, fungi, and bacteria, this chapter focuses on four major classes of plant alkaloids: the isoquinoline alkaloids, the terpenoid indole alkaloids, the tropane alkaloids, and the purine alkaloids.

In general, plant biosynthetic pathways are understood poorly when compared with prokaryotic and fungal metabolic pathways. A major reason for this poor understanding is that genes that express complete plant pathways typically are not clustered together on the genome. Therefore, each plant enzyme often is isolated individually and cloned independently. However, several enzymes involved in plant alkaloid biosynthesis have been cloned successfully, and many more enzymes have been purified from alkaloid-producing plants or cell lines (4–6). Identification and study of the biosynthetic enzymes has a significant impact on the understanding of the biochemistry of the pathway. Furthermore, genetic information also can be used to understand the complicated localization patterns and regulation of plant pathways. This chapter focuses on the biochemistry responsible for the construction of plant alkaloids and summarizes the biosynthetic genes that have been identified to date. Some of these pathways have been the subject of metabolic engineering studies; the results of these studies are mentioned here also. An excellent, more detailed review that covers the biochemistry and genetics of plant alkaloid biosynthesis up until the late 1990s is available also (7).

9.2 ISOQUINOLINE ALKALOIDS

The isoquinoline alkaloids include the analgesics morphine and codeine as well as the antibiotic berberine (Fig. 9.1a). Morphine and codeine are two of the most important analgesics used in medicine, and plants remain the main commercial source of the alkaloids (8). 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 (9).

9.2.1 Early Steps of Isoquinoline Biosynthesis

Isoquinoline biosynthesis begins with the substrates dopamine and *p*-hydroxyphenylacetaldehyde (Fig. 9.1b). Dopamine is made from tyrosine by hydroxylation and decarboxylation. Enzymes that catalyze the hydroxylation and decarboxylation steps in either order exist in the plant, and the predominant

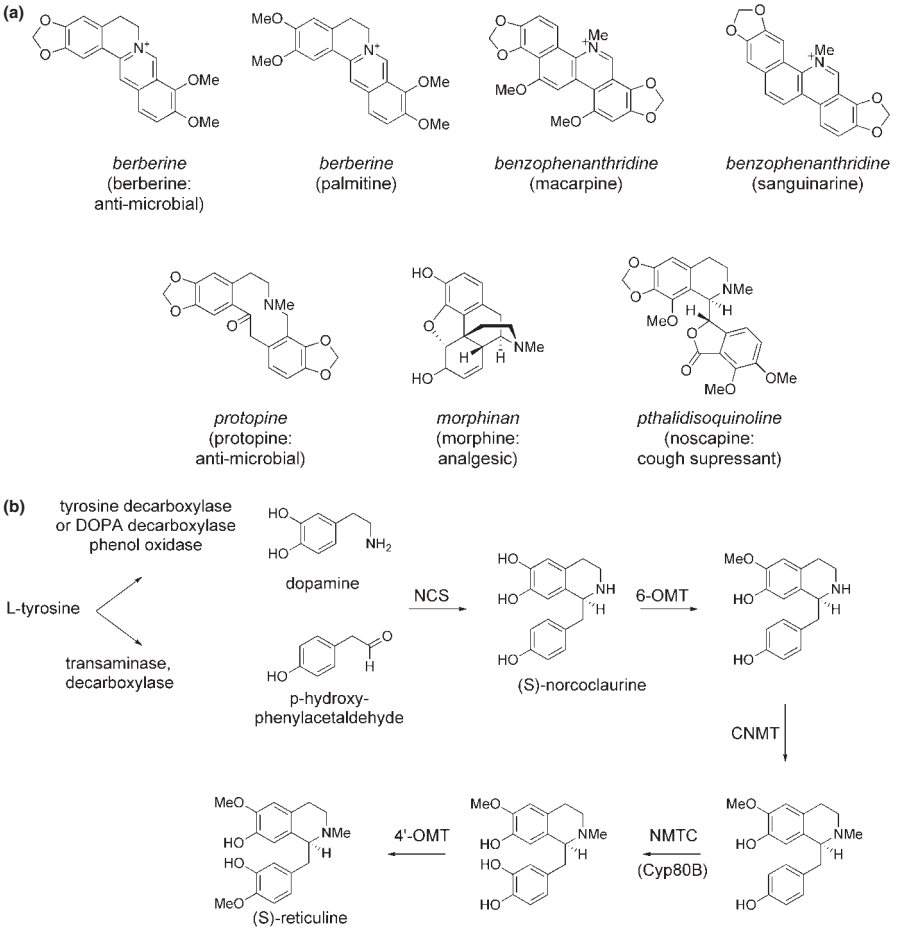


Figure 9.1 (a) Representative isoquinoline alkaloids. (b) Early biosynthetic steps of the isoquinoline pathway yield the biosynthetic intermediate (S)-reticuline, the central biosynthetic intermediate for all isoquinoline alkaloids. (c) Berberine and sanguinarine biosynthesis pathways. (d) Morphine biosynthesis. NCS, norcoclaurine synthase; 6-OMT, norcoclaurine 6-O-methyltransferase; CNMT, coclaurine N-methyltransferase (Cyp80B); NMTC, N-methylcoclaurine 3′-hydroxylase; 4′-OMT, 3′-hydroxy-N-methylcoclaurine 4′-O-methyltransferase; BBE, berberine bridge enzyme; SOMT, scoulerine 9-O-methyltransferase; CS, canadine synthase; TBO, tetrahydroprotoberberine oxidase; CHS, cheilanthifoline synthase; SYS, stylopine synthase; NMT, N-methyltransferase; NMSH, N-methylstylopine hydroxylase; P6H protopine 6-hydroxylase; DHPO, dihydrobenzophenanthridine oxidase; RO, reticuline oxidase; DHR, dihydroreticulinium ion reductase; STS, salutaridine synthase; SalR, salutaridine reductase; SalAT, salutaridinol acetyltransferase; COR, codeinone reductase.

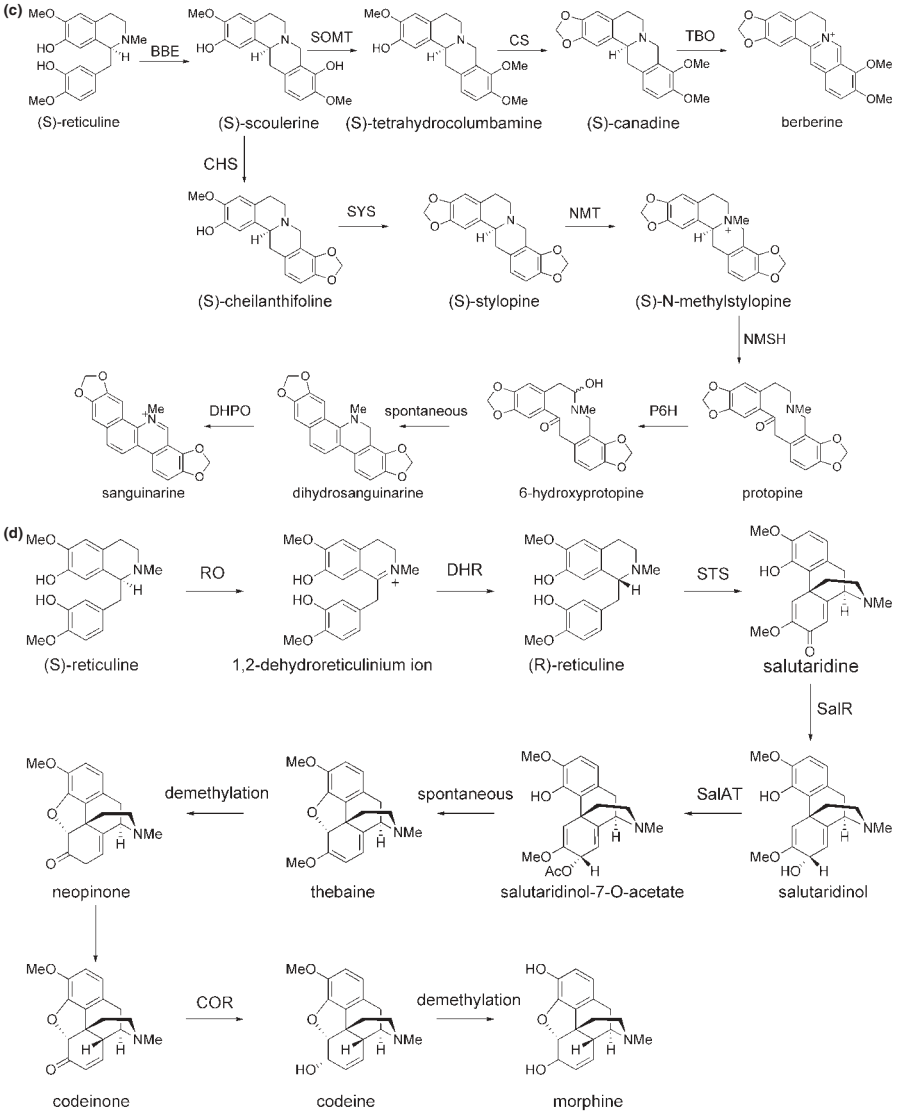


Figure 9.1 (*Continued*)

pathway for formation of dopamine from tyrosine is not clear. The second substrate, *p*-hydroxyphenylacetaldehyde, is generated by transamination and decarboxylation of tyrosine (10, 11).

Condensation of dopamine and *p*-hydroxyphenylacetaldehyde is catalyzed by norcoclaurine synthase to form (S)-norcoclaurine (Fig. 9.1b). Two norcoclaurine synthases with completely unrelated sequences were cloned (*Thalictrum flavum* and *C. japonica*) and heterologously expressed in *E. coli* (12–14). One

is homologous to iron-dependent diooxygenases, whereas the other is homologous to a pathogenesis-related protein. Undoubtedly, future experiments will shed light on the mechanism of these enzymes and on how two such widely divergent sequences can catalyze the same reaction.

One of the hydroxyl groups of (S)-norcoclaurine is methylated by a S-adenosyl methionine-(SAM)-dependent O-methyl transferase to yield (S)-coclaurine. This enzyme has been cloned, and the heterologously expressed enzyme exhibited the expected activity (15–17). The resulting intermediate is then N-methylated to yield N-methylcoclaurine, an enzyme that has been cloned (18, 19). N-methylcoclaurine, in turn, is hydroxylated by a P450-dependent enzyme (CYP80B), N-methylcoclaurine 3′-hydroxylase, that has been cloned (20, 21). The 4′ hydroxyl group then is methylated by the enzyme 3′-hydroxy-N-methylcoclaurine 4′-O-methyltransferase (4′-OMT) to yield (S)-reticuline, the common biosynthetic intermediate for the berberine, benzo(c)phenanthridine, and morphinan alkaloids (Fig. 9.1b). The gene for this methyl transferase also has been identified (15, 22). These gene sequences also were used to identify the corresponding *T. flavum* genes that encode the biosynthetic enzymes for reticuline from a cDNA library (23). At this point, the biosynthetic pathway then branches to yield the different structural classes of isoquinoline alkaloids.

9.2.2 Berberine Biosynthesis

(S)-reticuline is converted to (S)-scoulerine by the action of a well-characterized flavin-dependent enzyme, berberine bridge enzyme (Fig. 9.1c). This enzyme has been cloned from several plant species, and the mechanism of this enzyme has been studied extensively (24–28). (S)-scolerine is then O-methylated by scoulerine 9-O-methyltransferase to yield (S)-tetrahydrocolumbamine. Heterologous expression of this gene in *E. coli* yielded an enzyme that had the expected substrate specificity (29). A variety of O-methyl transferases also have been cloned from *Thalictrum tuberosum* (30). The substrate-specific cytochrome P450 oxidase canadine synthase (31) that generates the methylene dioxy bridge of (S)-canadine has been cloned (32). The final step of berberine biosynthesis is catalyzed by a substrate-specific oxidase, tetrahydroprotoberberine oxidase, the sequence of which has not been identified yet (33).

Overproduction of berberine in *C. japonica* cell suspension cultures was achieved by selection of a high-producing cell line (34) with reported productivity of berberine reaching 7 g/L (35). This overproduction is one of the first demonstrations of production of a benzylisoquinoline alkaloid in cell culture at levels necessary for economic production. This cell line has facilitated greatly the identification of the biosynthetic enzymes.

9.2.3 Sanginarine Biosynthesis

The biosynthesis of the highly oxidized benzo(c)phenanthidine alkaloid sanguinarine 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 (Fig. 9.1c) (36). Sanguinarine biosynthesis starts from (S)-scoulerine, as in berberine biosynthesis. Methylenedioxy bridge formation then is catalyzed by the P450 cheilanthifoline synthase to yield cheilanthifoline (37). A second P450 enzyme, stylopine synthase, catalyzes the formation of the second methyenedioxy bridge of stylopine (37). Stylopine synthase from *E. californica* has been cloned (38). Stylopine then is N-methylated by (S)-tetrahydroprotoberberine *cis*-N-methyltransferase to yield (S)-*cis*-N-methylstylopine, an enzyme that has been cloned from opium poppy (39). A third P450 enzyme, (S)-*cis*-N-methylstylopine hydroxylase, then forms protopine. Protopine is hydroxylated by a fourth P450 enzyme, protopine 6-hydroxylase, to yield an intermediate that rearranges to dihydrosanguinarine (40). This intermediate also serves as the precursor to the benzo(c)phenanthridine alkaloid macarpine (Fig. 9.1a). The copper-dependent oxidase dihydrobenzophenanthridine oxidase, which has been purified (41, 42), then catalyzes the formation of sanguinarine from dihydrosanguinarine.

Additional enzymes from other benzo(c)phenanthidine alkaloids have been cloned. For example, an O-methyl transferase implicated in palmitine biosynthesis has been cloned (43).

9.2.4 Morphine Biosynthesis

The later steps of morphine biosynthesis have been investigated in *P. somniferum* cells and tissue. Notably, in morphine biosynthesis, (S)-reticuline is converted to (R)-reticuline, thereby epimerizing the stereocenter generated by norcoclaurine synthase at the start of the pathway (Fig. 9.1d). (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 (44). 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 (45).

Next, the key carbon–carbon bond of the morphinan alkaloids is formed by the cytochrome P450 enzyme salutaridine synthase. Activity for this enzyme has been detected in microsomal preparations, but the sequence has not been identified (46). The keto moiety of the resulting product, salutaridine, then is stereoselectively reduced by the NADPH-dependent salutaridine reductase to form salutardinol. The enzyme has been purified (47), and a recent transcript analysis profile of *P. sominiferum* has resulted in the identification of the clone (48). Salutaridinol acetyltransferase, also cloned, then transfers an acyl group from acetyl-CoA to the newly formed hydroxyl group, which results in the formation of salutaridinol-7-O-acetate (49). This modification sets up the molecule to undergo a spontaneous reaction in which the acetate can act 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 (50, 51). Finally, codeine is demethylated by an uncharacterized enzyme to yield morphine.

The localization of isoquinoline biosynthesis has been investigated at the cellular level in intact poppy plants by using *in situ* RNA hybridization and immunoflouresence microscopy. The localization of 4′-OMT (reticuline biosynthesis), berberine bridge enzyme (saguinarine biosynthesis), salutaridinol acetyltransferase (morphine biosynthesis), and codeinone reductase (morphine biosynthesis) has been probed. 4′-OMT 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 (52). Another study, however, implicates a single cell type (sieve elements and their companion cells) in isoquinoline alkaloid biosynthesis (53, 54). 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. Localization of enzymes in alkaloid biosynthesis is difficult, and, undoubtedly, future studies will provide more insight into the trafficking involved in plant secondary metabolism.

9.2.5 Metabolic Engineering of Morphine Biosynthesis

In attempts to accumulate thebaine and decrease production of morphine (a precursor to the recreational drug heroine), codeinone reductase in opium poppy plant was downregulated by using RNAi (8). 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. However, the overexpression of codeinone reductase in opium poppy plants did result, in fact, in an increase in morphine and other morphinan alkaloids, such as morphine, codeine, and thebaine, compared with control plants (55). Gene expression levels in low morphine-producing poppy plants have been analyzed also (56). Silencing of berberine bridge enzyme in opium poppy plants also resulted in a change in alkaloid profile in the plant latex (57).

The cytochrome P450 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 (58). 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 P450 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.

9.3 TERPENOID INDOLE ALKALOIDS

The terpenoid indole alkaloids have a variety of chemical structures and a wealth of biologic activities (Fig. 9.2a) (59, 60). Terpenoid indole alkaloids are used as anticancer, antimalarial, and antiarrhythmic agents. Although many biosynthetic genes from this pathway remain unidentified, studies have correlated terpenoid indole alkaloid production with the transcript profiles of *Catharanthus roseus* cell cultures (61).

9.3.1 Early Steps of Terpenoid Indole Alkaloid Biosynthesis

All terpenoid indole alkaloids are derived from tryptophan and the iridoid terpene secologanin (Fig. 9.2b). Tryptophan decarboxylase, a pyridoxal-dependent enzyme, converts tryptophan to tryptamine (62, 63). The enzyme strictosidine synthase catalyzes a stereoselective Pictet–Spengler condensation between tryptamine and secologanin to yield strictosidine. Strictosidine synthase (64) has been cloned from the plants *C. roseus* (65), *Rauwolfia serpentine* (66), and, *Ophiorrhiza pumila* (67). A crystal structure of strictosidine synthase from *R. serpentina* has been reported (68, 69), and the substrate specificity of the enzyme can be modulated (70).

Strictosidine then is deglycosylated by a dedicated β-glucosidase, which converts it to a reactive hemiacetal intermediate (71–73). This hemiacetal opens to form a dialdehyde intermediate, which then forms dehydrogeissoschizine. The enol form of dehydrogeissoschizine undergoes 1,4 conjugate addition to produce the heteroyohimbine cathenamine (74–76). A variety of rearrangements subsequently act on deglycosylated strictosidine to yield a diversity of indole alkaloid products (77).

9.3.2 Ajmaline Biosynthesis

The biosynthetic pathway for ajmaline in *R. serpentina* is one of the best-characterized terpenoid indole alkaloid pathways. Much of this progress has been detailed in an extensive review (78). Like all other terpenoid indole alkaloids, ajmaline, an antiarrhythmic drug with potent sodium channel-blocking properties (79), is derived from deglycosylated strictosidine (Fig. 9.2c).

A membrane–protein fraction of an *R. serpentina* extract transforms labeled strictosidine (80, 81) into sarpagan-type alkaloids. The enzyme activity is dependent on NADPH and molecular oxygen, which suggests that sarpagan bridge enzyme may be a cytochrome P450 enzyme. Polyneuridine aldehyde esterase hydrolyzes the polyneuridine aldehyde methyl ester, which generates an acid that 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 (82, 83).

In the next step of the ajmaline pathway, vinorine synthase transforms the sarpagan alkaloid epi-vellosamine to the ajmalan alkaloid vinorine (84).

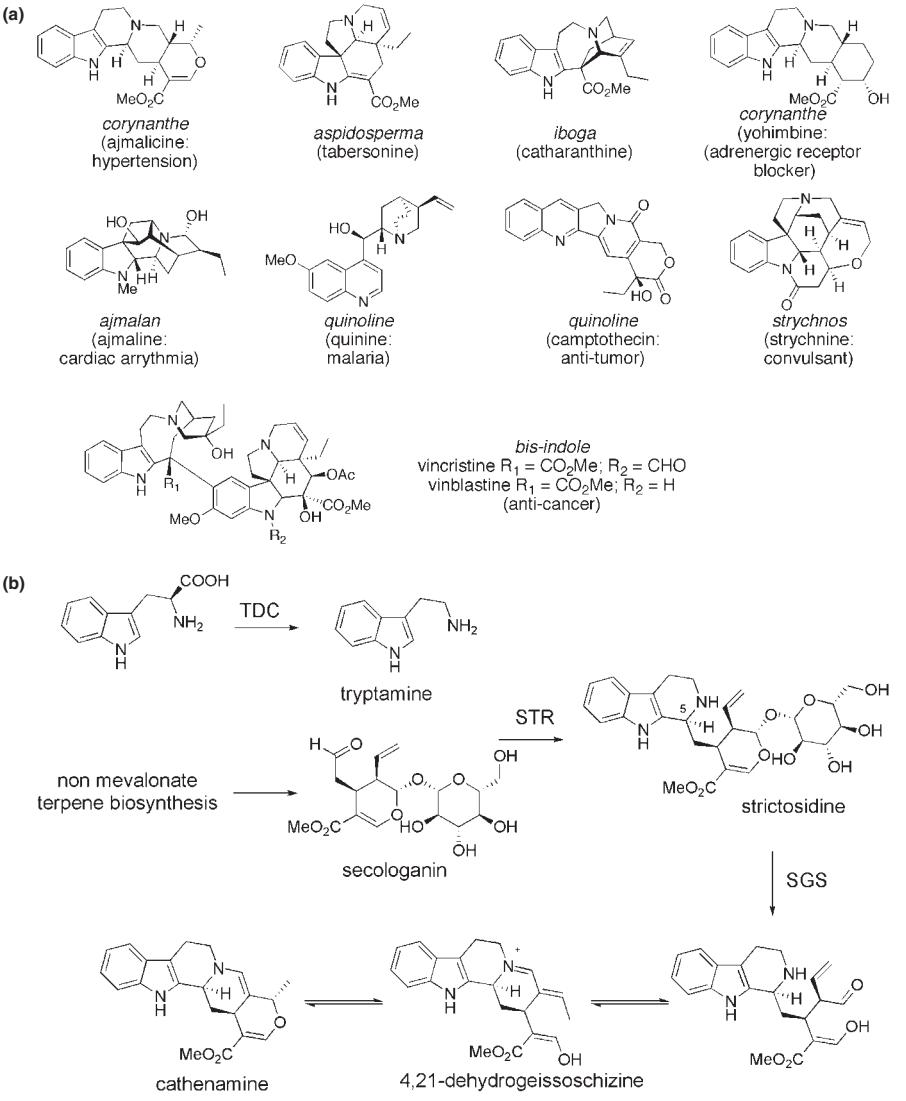
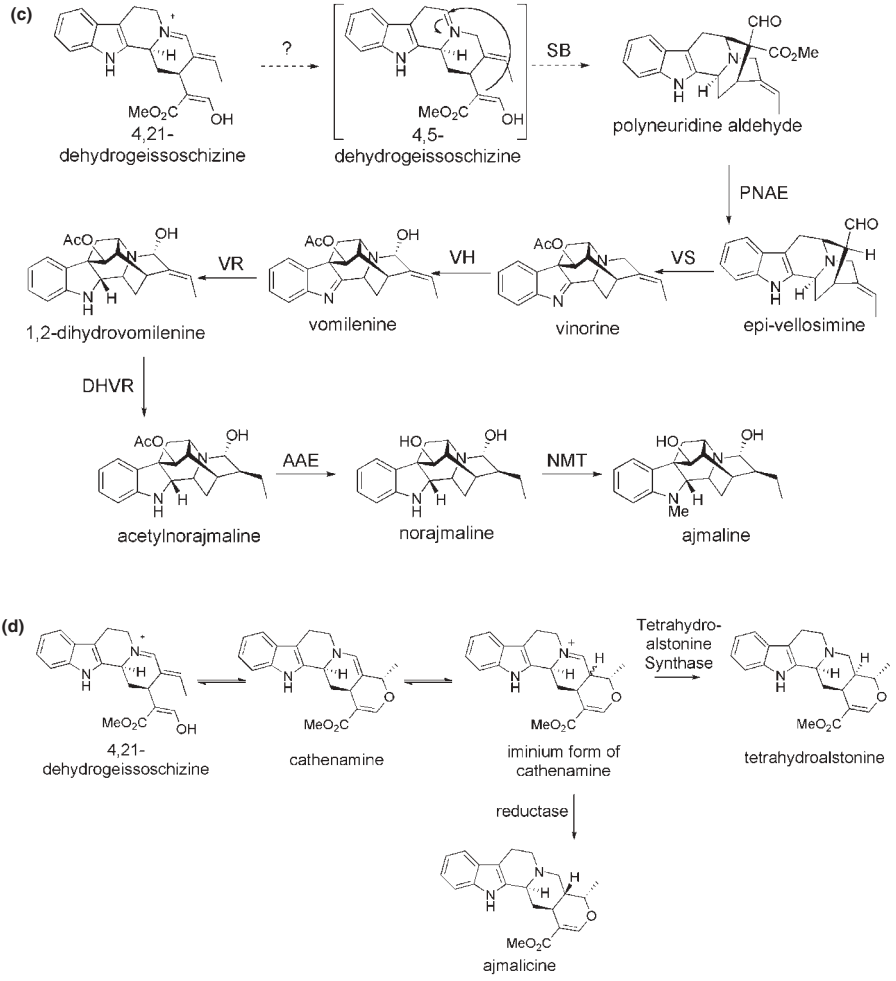


Figure 9.2 (a) Representative terpenoid indole alkaloids. (b) Early biosynthetic steps of the terpenoid indole alkaloid pathway yield the strictosidine, the central biosynthetic intermediate for all terpenoid indole alkaloids. (c) Ajmaline biosynthesis. (d) Ajmalicine and tetrahydroalstonine biosynthesis. (e) Vindoline biosynthesis from tabersonine. TDC, tryptophan decarboxylase; STR, strictosidine synthase; SGS, strictosidine glucosidase; SB, sarpagan bridge enzyme; PNAE, polyneuridine aldehyde reductase; VS, vinorine synthase; VH, vinorine hydroxylase; VR, vomilenine reductase; DHVR, dihydrovomilenine reductase; AAE, 17-O-acetyl-ajmalanesterase; NMT, norajmaline-N-methyltransferase; T16H, tabersonine-16-hydroxylase; HTOM, 16-hydroxytabersonine-16-O-methyltransferase; NMT, N-methyltransferase; D4H, desacetoxyvindoline-4-hydroxylase; DAT, desacetylvindoline O-acetyltransferase.



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Vinorine synthase also has been purified from *Rauwolfia* cell culture, subjected to protein sequencing, and cloned from a cDNA library (85, 86). The enzyme, which seems to be an acetyl transferase homolog, has been expressed heterologously in *E. coli*. Crystallization and site-directed mutagenesis studies of this protein have led to a proposed mechanism (87).

Vinorine hydroxylase hydroxylates vinorine to form vomilene (88). Vinorine hydroxylase seems to be a P450 enzyme that requires an NADPH-dependent reductase. This enzyme is labile and has not been cloned yet. Next, the indolenine bond 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

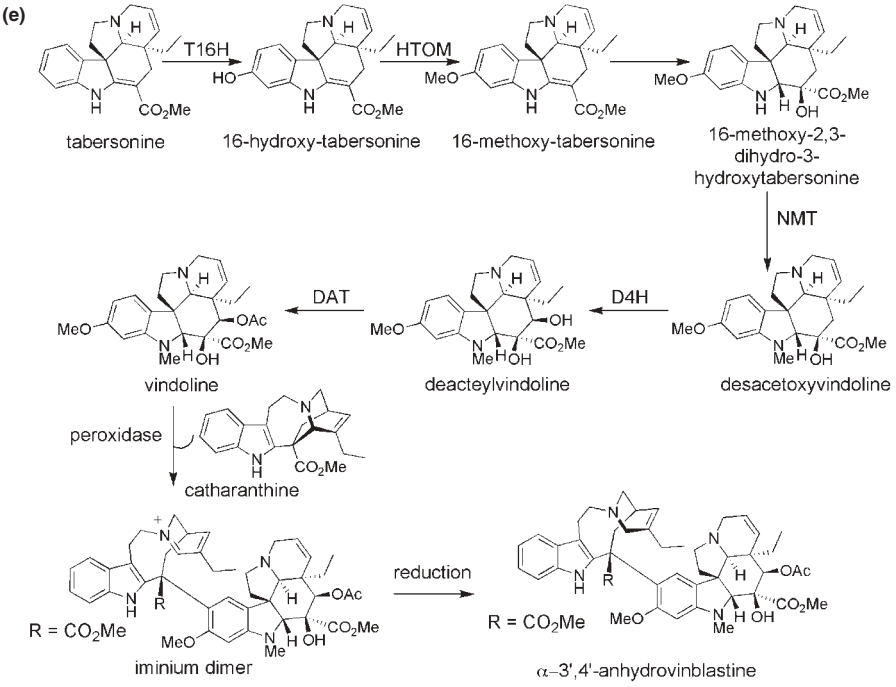

Figure 9.2 (*Continued*)

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 (89, 90).

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 (91). In the final step of ajmaline biosynthesis, an N-methyl transferase 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 (92).

9.3.3 Ajmalicine and Tetrahydroalstonine

Ajmalicine (raubasine) affects smooth muscle function and is used to help prevent strokes (93), and tetrahydroalstonine exhibits antipsychotic properties (Fig. 9.2d) (94). 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* (95). 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 NADPD support a mechanism in which the reductase acts on the iminium form of cathenamine (96).

9.3.4 Vindoline

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*. Vindoline, like ajmalicine and ajmaline, is produced from deglycosylated strictosidine. Deglycosylated strictosidine is converted to tabersonine through a series of biochemical steps for which no enzymatic information exists. More details are known about the six steps that catalyze the elaboration of tabersonine to vindoline (Fig. 9.2e) (97).

Tabersonine-16-hydroxylase, a cytochrome P450, hydroxylates tabersonine to 16-hydroxy-tabsersonine in the first step of this sequence and has been cloned (98, 99). The newly formed hydroxyl group is methylated by a SAM-dependent O-methyl transferase to yield 16-methoxy-tabersonine; this enzyme (16-hydroxytabersonine-16-O-methyltransferase) has been purified but not cloned (100). 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-methyl transferase yields desacetoxyvindoline. This methyl transferase activity has been detected only in differentiated plants, not in plant cell cultures (101). The resulting intermediate, deacteylvindoline, is produced by the oxoglutatarate-dependent dioxygenase enzyme desacetylvindoline 4-hydroxylase. This enzyme has been cloned and also is absent from plant cell cultures (102). In the last step, desacteylvindoline is acetylated by desacteylvindoline O-acetyl transferase. This enzyme, also absent from nondifferentiated plant material, has been cloned successfully (103).

As in morphine biosynthesis, the knowledge of the enzyme sequences allows a more detailed understanding of the localization of the enzymes (104). Strictosidine synthase (Fig. 9.2b) seems to be localized to the vacuole (105), and strictosidine glucosidase is believed to be associated with the membrane of the endoplasmic reticulum (73, 106). Tabersonine-16-hydroxylase is associated with the endoplasmic reticulum membrane (98); N-methyl transferase activity is believed to be associated with the thykaloid, a structure located within the chloroplast (101, 107); and vindoline-4-hydroxylase and desacetylvindoline O-acetyltransferase are believed to be localized to the cytosol (Fig. 9.2e) (107, 108). Overall, extensive subcellular trafficking of biosynthetic intermediates is required for vindoline biosynthesis.

Aside from subcellular compartmentalization, specific cell types are required for the biosynthesis of some terpenoid alkaloids. 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 (109). However, additional studies have suggested that these genes also are observed in the epidermis and laticifers (110). Studies of the localization of vindoline biosynthetic enzymes by using immunocytochemistry and *in situ* RNA hybridization strongly suggest that the mid-part 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 (109–112). As with isoquinoline alkaloid biosynthesis, deconvolution of the enzyme localization patterns remains a challenging endeavor.

9.3.5 Vinblastine

Vinblastine is a highly effective anticancer agent currently used clinically against leukemia, Hodgkin's lymphoma, and other cancers. (113, 114). Vinblastine is derived from dimerization of vindoline and another terpenoid indole alkaloid, catharanthine. The dimerization of catharanthine and vindoline is believed to proceed via the formation of an iminium intermediate with catharanthine (Fig. 9.2e). This iminium intermediate is reduced to form anhydrovinblastine, a naturally occurring compound in *C. roseus* plants (115). In support of this mechanism, anhydrovinblastine is incorporated into vinblastine and vincristine in feeding studies (116–119).

Peroxidase containing fractions of plant extracts were found to catalyze the formation of the bisindole dehydrovinblastine from catharanthine and vindoline (120, 121). A peroxidase from *C. roseus* leaves has been demonstrated to convert vindoline and catharanthine to anhydrovinblastine *in vitro* (122, 123). Because the dimerization of these *C. roseus* alkaloids also can be catalyzed by peroxidase from horseradish in reasonable yields (124), it is interesting to speculate that anhydrovinblastine may be a by-product of isolation; after lysis of the plant material, nonspecific peroxidases are released from the vacuole and may act on vindoline and catharanthine.

9.3.6 Metabolic Engineering of Terpenoid Indole Alkaloids

Strictosidine synthase and tryptophan decarboxylase have been overexpressed in *C. roseus* cell cultures (125, 126). 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 (127, 128). Secologanin biosynthesis seems to be the rate-limiting factor in alkaloid production (129). Precursor-directed biosynthesis experiments with a variety of tryptamine analogs suggest that the biosynthetic pathway can be used to produce alkaloid derivatives (130). Strictosidine synthase and strictosidine glucosidase enzymes also have been expressed successfully heterologously

in yeast (131); however, 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 (132), as well as a transcription factor that coordinately upregulates expression of several terpenoid indole alkaloid biosynthetic genes, have been found (133). Several zinc finger proteins that act as transcriptional repressors to tryptophan decarboxylase and strictosidine synthase also have been identified (134). 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 (135).

9.4 TROPANE ALKALOIDS

The tropane alkaloids hyoscyamine and scopolamine (Fig. 9.3a) function as acetylcholine receptor antagonists and are used clinically as parasympatholytics. The illegal drug cocaine also is a tropane alkaloid. The tropane alkaloids are biosynthesized primarily in plants of the family *Solonaceae*, which includes *Hyoscyamus*, *Duboisia*, *Atropa*, and *Scopolia* (136, 137). Nicotine, although perhaps not apparent immediately from its structure, is related biosynthetically to the tropane alkaloids (Fig. 9.3b).

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 (Fig. 9.3b) (138). 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 serves as the common precursor for the tropane alkaloids.

Putrescine is N-methylated by a SAM-dependent methyl transferase that has been cloned to yield N-methylputrescine (139, 140). Putrescine N-methyl transferase now has been cloned from a variety of plant species (141–143), and site-directed mutagenesis and homology models have led to insights into the structure function relationships of this enzyme (143). N-methylputrescine then is oxidized by a diamine oxidase to form 4-methylaminobutanal, which then spontaneously cyclizes to form the N-methyl-D-pyrrolinium ion (144, 145). This enzyme, which has been cloned, seems to be a copper-dependent amine oxidase (146, 147). Immunoprecipitation experiments suggest that this enzyme associates with the enzyme S-adenosylhomocysteine hydrolase (148). The pyrrolinium ion then is converted to the tropanone skeleton by as yet uncharacterized enzymes (Fig. 9.3b). Although no enzymatic information is available, chemical labeling studies have indicated that an acetate-derived moiety condenses with the pyridollium ion; one possible mechanism is shown in Fig. 9.3b (136).

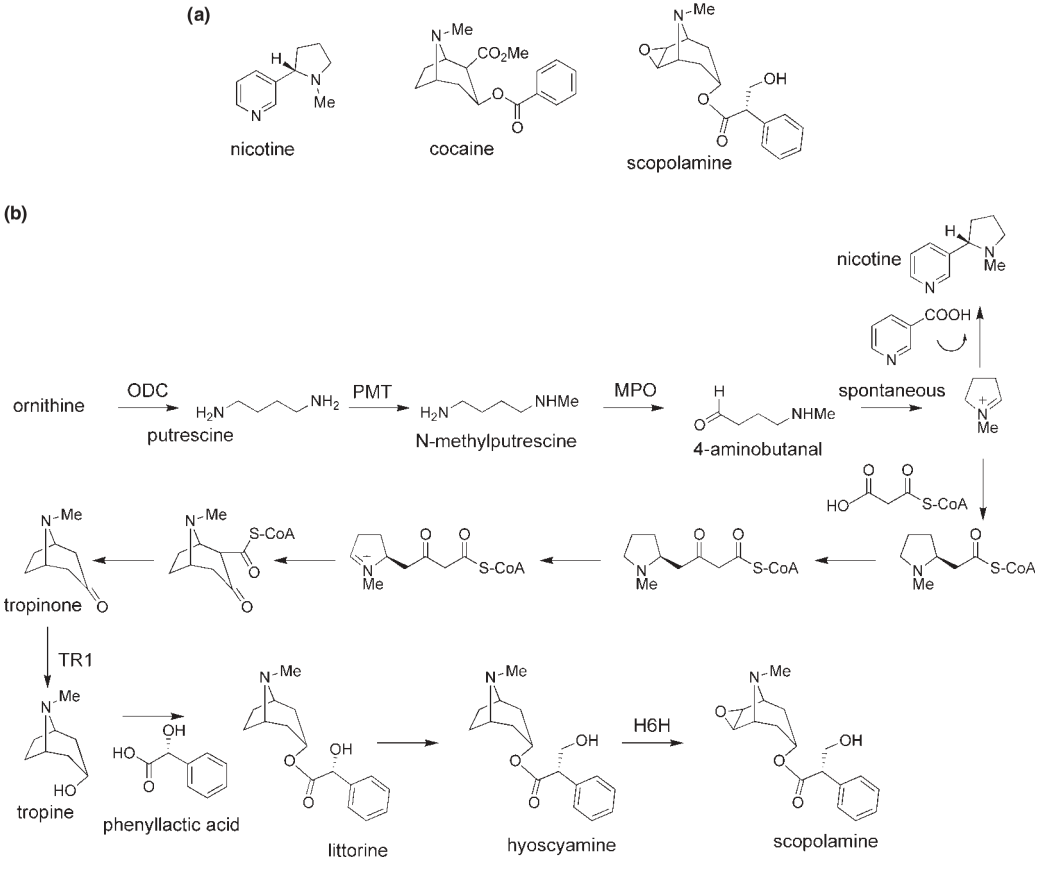

Figure 9.3 (a) Representative tropane and nicotine alkaloids. (b) Tropane biosynthesis. ODC, ornithine decarboxylase; PMT, putrescine N-methyltransferase; MPO, diamine oxidase; TR1, tropinone reductase 1; H6H, hyocyamine 6b-hydroxylase.

Tropanone then is reduced via an NADPH-dependent reductase to tropine that has been cloned from *Hyoscyamus niger* (149, 150). 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 (Fig. 9.3b), whereas tropinone reductase II yields the opposite stereocenter, pseudotropine (151). Tropane is converted to scopolamine or hyoscyamine, whereas the TRII product pseudotropine leads to calystegines (152). These two tropinone reductases have been crystallized, and site-directed mutagenesis studies indicate that the stereoselectivity of the enzymes can be switched (153, 154).

The biosynthesis of scopolamine is the best characterized of the tropane alkaloids. After action by tropinone reductase I, tropine is condensed with phenyllactate through the action of a P450 enzyme to form littorine (155). The phenyllactate moiety is believed to derive from an intermediate involved in phenylalanine metabolism (136). Littorine then undergoes rearrangement to form hyoscyamine. The enzyme that catalyzes this rearrangement, which has been purified partially, seems to proceed via a radical mechanism using S-adenysylmethione as the source of an adenosyl radical (156). Labeling studies have been used to examine the mechanism of rearrangement (136)(157–159). Hyoscyamine 6β-hydroxylase (H6H) catalyzes the hydroxylation of hyoscyamine to 6β-hydroxyhyoscyamine as well as the epoxidation to scopolamine (Fig. 9.3b) (160, 161). H6H, which has been cloned and expressed heterologously (162), is a nonheme, iron-dependent, oxoglutarate-dependent protein. It seems that the epoxidation reaction occurs much 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 (163).

9.4.1 Metabolic Engineering of Tropane Alkaloids

Atropa belladonna plants have been transformed with an H6H clone from *H. niger*. *A. belladonna* normally produces high levels of hyoscyamine, the precursor for the more pharmaceutically valuable alkaloid scopolamine (Fig. 9.3b). However, after transformation with the H6H gene, transgenic *A. belladonna* plants were shown to accumulate scopolamine almost exclusively (164). Additionally, the levels of tropane alkaloid production in a variety of hairy root cultures were altered by overexpression of methyltransferase putrescine-N-methyltransferase and H6H. Overexpression of both of these enzymes in a hairy root cell culture resulted in significant increases in scopolamine production (164, 165). Fluorinated phenyllactic acid substrates could be incorporated into the pathway (166), and several substrates derived from putrescine analogs were turned over by the enzymes of several *Solonaceae* species (167).

9.5 PURINE ALKALOIDS

9.5.1 Caffeine Biosynthesis

Caffeine, a purine alkaloid, is one of the most widely known natural products. Caffeine is ingested as a natural component of coffee, tea, and cocoa, and the

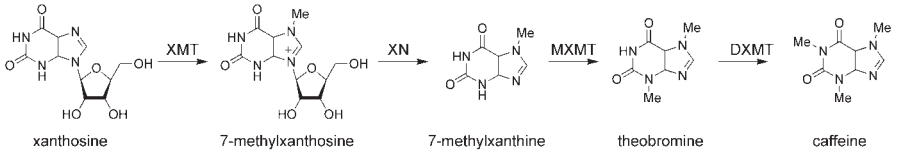

Figure 9.4 Caffeine biosynthesis. XMT, xanthosine N-methyltransferase (also called 7-methylxanthosine synthase); XN, methylxanthosine nucleotidase; MXMT, 7-methylxanthine-N-methyltransferase (also called theobromine synthase); DXMT, dimethylxanthine-N-methyltransferase (also called caffeine synthase).

impact of caffeine on human health has been studied extensively. The biosynthetic pathway of caffeine has been elucidated on the genetic level. Caffeine biosynthesis has been studied most widely in the plant species *Coffea* (coffee) and *Camellia* (tea) (168, 169).

Xanthosine, which is derived from purine metabolites, is the first committed intermediate in caffeine biosynthesis (Fig. 9.4). Xanthosine can be formed from *de novo* purine biosynthesis, S-adenosylmethione (SAM) cofactor, the adenylate pool, and the guanylate pool (169). *De novo* purine biosynthesis and the adenosine from SAM are believed to be the most important sources of xanthosine (168, 170).

The biosynthesis of caffeine begins with the methylation of xanthosine to yield N-methylxanthosine by the enzyme xanthosine N-methyltransferase (XMT) (also called 7-methylxanthosine synthase) (171–173). N-methylxanthosine is converted to N-methylxanthine by methylxanthine nucleosidase, an enzyme that has not been cloned yet (174). N-methylxanthine is converted to theobromine by 7-methylxanthine-N-methyltransferase (MXMT) (also called theobromine synthase), a second N-methyltransferase (171, 175). Theobromine is converted to caffeine by a final N-methyltransferase, dimethylxanthine-N-methyltransferase (DXMT) (also called caffeine synthase) (171).

Coffee and tea plants seem to contain a variety of N-methyltransferase enzymes that have varying substrate specificity (168, 169). For example, a caffeine synthase enzyme isolated from tea leaves catalyzes both the N-methylation of N-methylxanthine and theobromine (176). The substrate specificity of the methyltransferases can be changed by site-directed mutagenesis (177), and the crystal structure of two of the N-methyltransferases has been reported (178).

9.5.2 Metabolic Engineering of Caffeine Biosynthesis

Caffeine may act as a natural insecticide in plants. When the three N-methyltransferase genes were overexpressed in tobacco, the resulting increase in caffeine production improved the tolerance of the plants to certain pests (179). Conversely, coffee beans with low caffeine levels would 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 (180, 181). For example, a 70% reduction in caffeine content in *Coffea* was obtained by downregulating MXMT (theobromine synthase) using RNAi (182). Additionally, the promoter of one of the N-methyltransferases has been discovered, which may allow transcriptional gene silencing (183).

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