CHAPTER 8

Aromatic and Phenolic Compounds

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8.1 Overview

Plants are autotrophic organisms and must synthesize all their metabolites from the intermediates of the C-3 cycle and other pathways of primary carbon and nitrogen metabolism. This includes the 20 common amino acids used in protein synthesis. Many of these amino acids are synthesized directly from precursors derived from primary metabolism. For example, the amino acid glutamic acid is produced in a single reaction: transfer of an amino group to 2-oxoglutarate (α-ketoglutaric acid), an intermediate in the citric acid cycle. Synthesis of the aromatic amino acids, phenylalanine, tyrosine, and tryptophan, is more complex. In addition to their function in protein synthesis, most amino acids serve as precursors for the synthesis of other metabolites. This is especially true for phenylalanine, which is the precursor for the phenylpropanoid pathway. Removal of the amino group from phenylalanine yields *trans*-cinnamic acid followed by addition of an OH and coenzyme A, which produces *p*-coumaroyl-CoA. This key intermediate is essential in synthesis of lignin precursors and is considered a primary metabolic pathway in terrestrial plants. Coumaroyl-CoA is also the initial compound for a variety of natural products, including flavonoid synthesis.

Flavonoids are found in most plants and give rise to colored compounds such as anthocyanins. They function to protect plants from intense light and ultraviolet (UV) radiation and can act as antioxidants. A vast number of additional natural products are synthesized from the aromatic amino acids and their precursors. Only a small number of these are presented in this chapter.

8.2 Shikimic Acid Pathway

All three aromatic amino acids are derived from intermediates of the same series of reactions, the shikimic acid pathway. Synthesis starts with erythrose-4-phosphate and phosphoenolpyruvic acid (PEP). The pathway is named after the first intermediate that is unique to aromatic amino acid synthesis, shikimic acid. The reactions in this pathway are similar in plants, fungi, and bacteria but are absent in animals. The component enzymes are homologous in all these kingdoms. In plants, the enzymes are found in plastids and are presumably all soluble in the stroma. The genes for these enzymes are encoded in the nucleus. An overview of the pathway is shown in **FIGURE 8.1**. In addition to the three aromatic amino acids, the intermediates of the shikimic acid pathway are also used in the synthesis of a variety of other metabolites, including the plant hormone salicylic acid, vitamins such as folic acid, and a number of species-specific natural products.

The erythrose-4-phosphate precursor is an intermediate in the C-3 cycle and the pentose phosphate pathway in the chloroplast stroma (see Chapters 2 and 3). PEP is an intermediate in glycolysis and may be imported into chloroplasts. Alternatively, it can be a product of chloroplast pyruvate metabolism via pyruvate kinase and pyruvate, Pi dikinase. The first reaction in the pathway is an aldol condensation that results in the synthesis of a 7-carbon ketose, 3-deoxy-arabino-heptulosonic acid-7-phosphate (DAHP), shown in **FIGURE 8.2**.

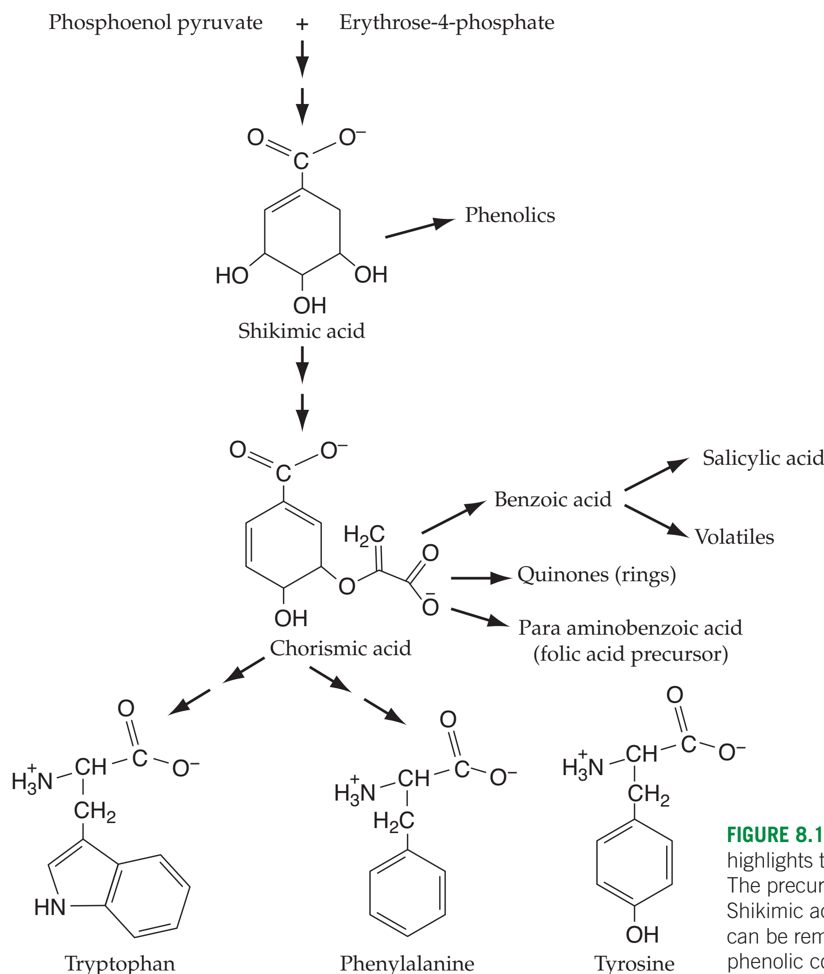

FIGURE 8.1 Simplified overview of the shikimic acid pathway. This diagram highlights the main intermediates and products of the shikimic acid pathway. The precursors are phosphoenolpyruvate (PEP) and erythrose-4-phosphate. Shikimic acid and chorismic acid are key intermediates. These intermediates can be removed from the pathway and used to synthesize a variety of additional phenolic compounds.

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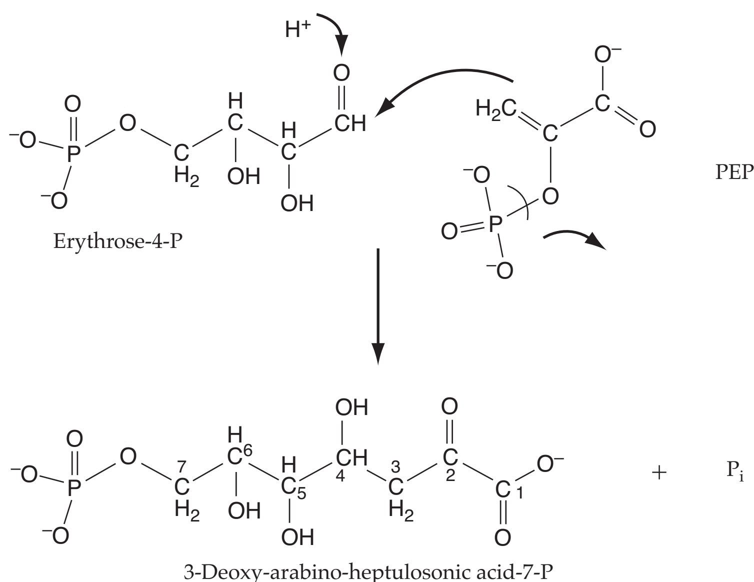

FIGURE 8.2 Synthesis of 3-deoxy-arabino-heptulosonic acid-7-phosphate (DAHP). The DAHP synthase catalyzes an aldol-type condensation. The phosphate is removed from PEP, and its methylene carbon then forms a bond with the carbonyl carbon of erythrose-4-phosphate.

DAHP synthases in plants and bacteria show only about 20% amino acid identity. The plant enzyme, however, complements bacterial mutants lacking this synthase. The bacterial enzyme exhibits feedback inhibition by the aromatic amino acid end products, phenylalanine and tyrosine. This does not appear to be true of the plant synthases. Expression of different isozymes of DAHP synthase in plants, however, is influenced by environmental factors such as high light intensity or wounding and the presence of hormones, gibberellic acid and jasmonic acid. The intermediates and end products of the shikimic acid pathway are often intermediates in the production of phytoalexins and other components of plant defense reactions.

The second step in the pathway is the formation of a cyclic intermediate, 3-dehydroquinic acid, from DAHP (**FIGURE 8.3**). The enzyme, dehydroquinate synthase, is an oxidoreductase that requires NAD as a cofactor. The enzyme in this case catalyzes both an oxidation followed by a reduction, thereby regenerating the oxidized cofactor.

Dehydroquinic acid is the substrate for a dehydratase that catalyzes removal of water and introduces a double bond into the ring. This is followed by reduction of the ketone to an alcohol forming shikimic acid, the first unique intermediate (**FIGURE 8.4**). In some plants, shikimic acid is the starting material for the synthesis of a variety of phenolic natural products. The most common are the water-soluble gallotannins, which are complexes of phenolics with sugars, usually glucose. Plants make these compounds to protect their tissues from UV damage and to deter herbivores. Tannins can bind irreversibly to proteins and inhibit enzyme activity. Humans have traditionally used tannic acid–rich plant extracts to tan and preserve animal hides. Some humans also like the bitter flavor of gallotannins in beverages such as tea.

Shikimic acid is further metabolized to chorismic acid, another key intermediate in this pathway. A kinase adds a phosphate group to one of the meta hydroxyls to produce shikimic acid-3-phosphate. This intermediate is then condensed with another molecule

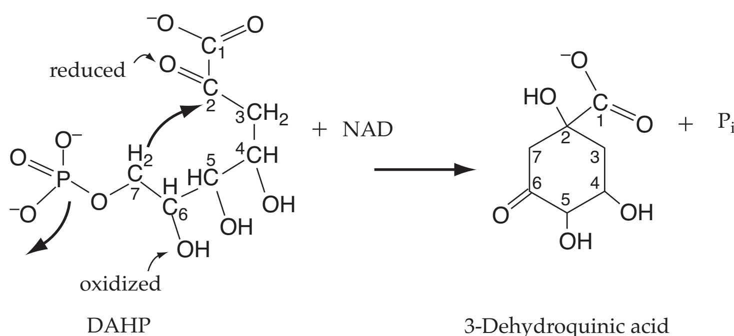

FIGURE 8.3 Reactions catalyzed by dehydroquinate synthase. The phosphate is removed from C-7 of DAHP and the resulting carbocation forms a bond with C-2. The enzyme then catalyzes the oxidation of the hydroxyl (OH) group on C-6 to a ketone and a reduction of the ketone on C-2 to an OH to yield 3-dehydroquinic acid. The redox cofactor for this enzyme is NAD, which is regenerated during the reaction.

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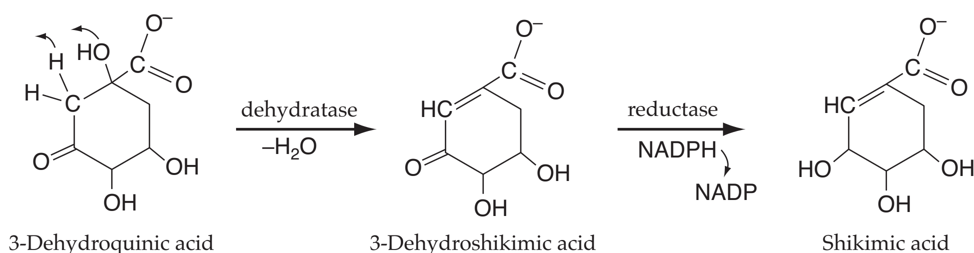

FIGURE 8.4 Synthesis of shikimic acid. A dehydratase-reductase binds dehydroquinic acid. It catalyzes removal of the hydroxyl next to the carboxyl group on C-1 and a proton from the adjacent C-6 carbon, generating a double bond in the ring. The NADPH-dependent reductase activity then reduces the keto group to a hydroxyl group to generate shikimic acid.

of PEP to produce 5-enolpyruvyl-shikimate-3-phosphate (EPSP) (**FIGURE 8.5**). The enzyme, EPSP synthase, is inhibited by an amino acid analog, *N*-phosphonomethyl glycine, also known as glyphosate. Although the structures do not appear to be similar, glyphosate competes with PEP for the same binding site on the synthase. As shown in **FIGURE 8.6**, the synthase consists of two globular domains. Binding of shikimate-3-phosphate triggers a global

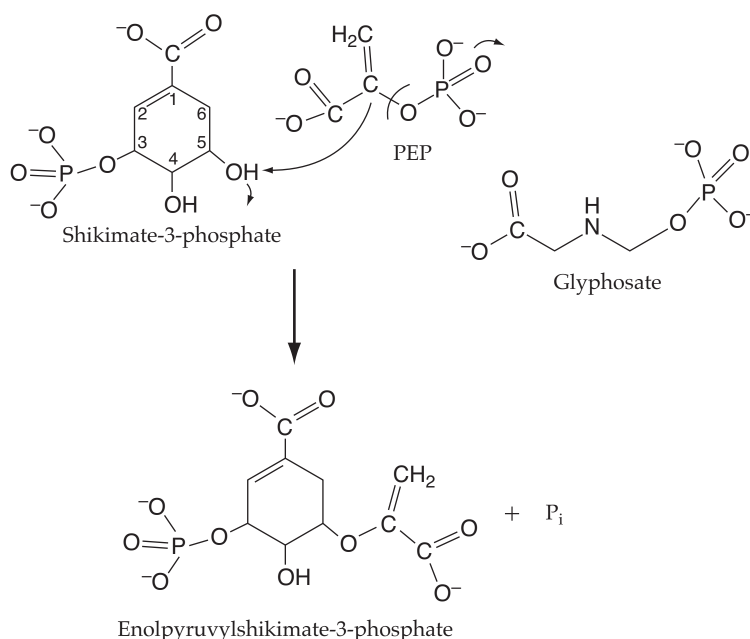

FIGURE 8.5 Synthesis of 5-enol-pyruvyl-shikimate-3-phosphate (EPSP). The EPSP synthase catalyzes removal of the phosphate from PEP. The resulting three-carbon fragment is added to the hydroxyl group on C-3 of shikimate-3-phosphate. The amino acid analog, glyphosate (inset), is a competitive inhibitor of PEP.

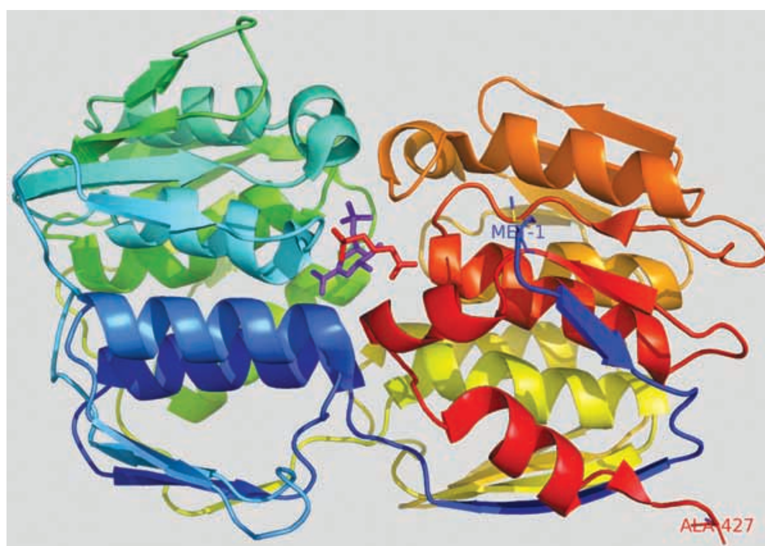

FIGURE 8.6 Structure of EPSP synthase. The enzyme consists of two domains shown in blue-green and red-yellow. In the absence of substrate, a wide gap separates the two domains. After binding shikimate-3-phosphate (shown as purple sticks), the enzyme undergoes a large conformational shift to narrow the gap between the domains as shown. The constricted active site can now bind pyruvate or glyphosate, shown as red sticks. The EPSP synthase is a good example of "induced fit" where binding of one substrate induces a conformational change in the protein to accommodate the second substrate and subsequent catalysis. (Structure from Protein Data Bank 1G6S. E. Schonbrunn, S. Eschenburg, W. Shuttleworth, J. V. Schloss, N. Amrhein, J. N. S. Evans, and W. Kabsch, *Proc. Natl. Acad. Sci.* 98 [2001]: 1376–1380.)

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conformational change to the more closed structure seen in Figure 8.6. PEP then binds to the active site formed at the interface of the two domains. Both kinetic and crystal structure analyses show that glyphosate competes with PEP for this same site. The unique nature of this structural change is further illustrated by the fact that glyphosate does not compete with PEP in any of the many other reactions that use PEP as a substrate. Glyphosate is a highly effective, broad-spectrum herbicide and is the active component in Monsanto's Roundup®.

EPSP is the substrate for a synthase that removes the phosphate from C-3 and introduces a second double bond into the ring. This results in the production of chorismic acid (**FIGURE 8.7**). Chorismic acid can be a precursor for benzoic acid and its derivatives and also salicylic acid in some plants. Methylated benzoic and salicylic acids are volatile and are responsible for many floral scents that attract pollinators. Salicylic acid is also a signaling molecule that initiates responses to abiotic stress and pathogen invasion, a process referred to as systemic acquired resistance (SAR). Additionally, chorismic acid in some plants provides the ring portion of quinones and *para*-amino benzoic acid, the precursor for folic acid synthesis. In many plants some of these compounds can also be derived from phenylalanine.

Phenylalanine and Tyrosine Synthesis

Chorismic acid is the branch point between synthesis of phenylalanine and tyrosine or tryptophan (Figure 8.1). Chorismate mutase catalyzes the transfer of the pyruvyl side chain to C-1 to produce prephenic acid, the precursor of phenylalanine and tyrosine (Figure 8.7). This mutase is allosterically regulated by the three aromatic amino acids. Allosteric regulators are generally small molecules that can either activate or inhibit enzyme activity, but these regulators do not bind to the enzyme's active site. Most allosterically regulated enzymes are composed of two or more subunits, and the regulators generally act by changing the subunit association. In the case of chorismate mutase, the enzyme's activity is inhibited by phenylalanine and tyrosine and activated by tryptophan, thereby ensuring balanced pools of all three amino acids.

In the next step, an amino group is added to prephenic acid to produce arogenic acid (Figure 8.7). The transaminase that catalyzes

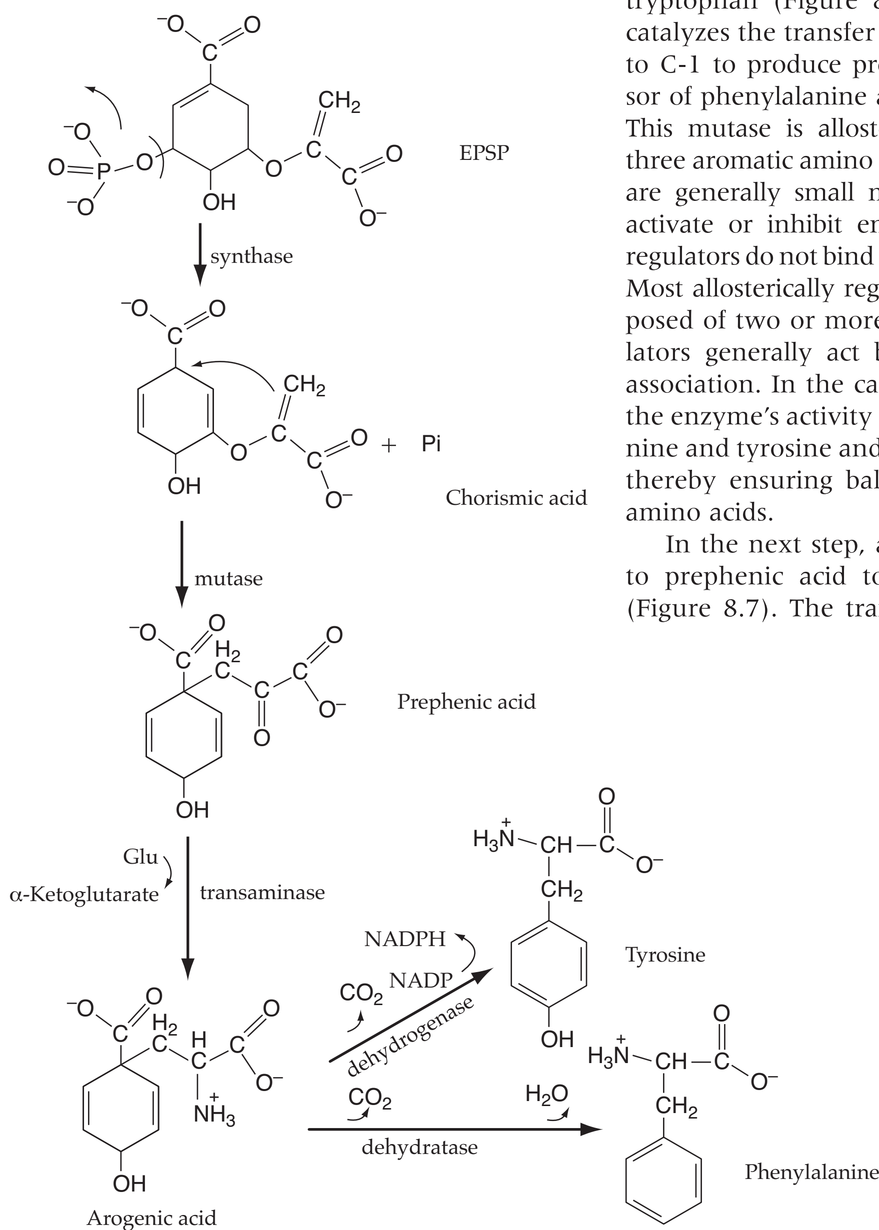

FIGURE 8.7 Synthesis of tyrosine and phenylalanine from chorismic acid. Chorismate synthase catalyzes the removal of the phosphate group from EPSP and introduces a second double bond into the ring to generate chorismic acid. The prephenate mutase reaction results in the relocation of the pyruvyl moiety to C-1 of the ring producing prephenic acid. A transaminase then catalyzes transfer of an amino group from glutamic acid to C-2 of the side chain resulting in the amino acid, arogenic acid. In most plants, arogenic acid is the precursor for both tyrosine and phenylalanine. To produce tyrosine, an NADP-dependent dehydrogenase oxidatively decarboxylates C-1 of the ring and catalyzes formation of a third double bond to generate an aromatic ring. In phenylalanine synthesis, a dehydratase removes both the C-1 carboxyl group and a water molecule from the ring, thereby generating the aromatic amino acid.

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this reaction uses glutamic acid as the amino donor. Oxidative-decarboxylation of arogenic acid by an NADP-dependent dehydrogenase yields tyrosine. To synthesize phenylalanine, decarboxylation of arogenic acid is followed by a dehydration to produce the aromatic ring.

Tryptophan Synthesis

Chorismic acid is also the precursor for the synthesis of tryptophan (Figure 8.1). A synthase catalyzes removal of the pyruvyl side chain and a transamination to produce anthranilic acid (**FIGURE 8.8**). The amino donor in this reaction is glutamine. The next step involves a glycosylation, that is, addition of 5-phospho-ribosyl-1-diphosphate to the amino group of anthranilic acid. The sugar-diphosphate is a good leaving group, and the sugar is bonded to anthranilate by an *N*-glycosidic bond as seen in nucleosides. An isomerase then catalyzes the formation of the ketose derivative. Decarboxylation of the aromatic ring and dehydration and cyclization produce the five-membered ring of indole glycerol-phosphate.

The final step in this branch of the pathway is catalyzed by tryptophan synthase. This enzyme cleaves the glycerol-P moiety from the indole ring and replaces it with serine (Figure 8.8). Tryptophan synthase is an $\alpha_2\beta_2$ heterotetramer in plants. It is an unusual example of a bifunctional enzyme. Indole glycerol-phosphate binds to an active site located on the α subunit. Binding causes a conformational change that closes the active site. After removal of glyceraldehyde-3-phosphate, the indole ring diffuses through a long protein tunnel (~25Å) between subunits to the active site in the β subunit. Serine then binds and closes the active site in the β subunit. The hydroxyl group is removed from serine, and the beta-carbon is bonded to the indole ring. Formation of tryptophan opens the active sites on both subunits, and the products are released. Although crystal structures of several tryptophan synthases with bound intermediates are available, it is still not known how the two active sites communicate to coordinate their activity, but this enzyme does not produce free indole. Some plants, such as maize, produce indole-based phytoalexins. In this case an enzyme resembling only the α subunit of tryptophan synthase is found in the cytoplasm. A similar enzyme is the most likely source of the volatile indole released from many plants.

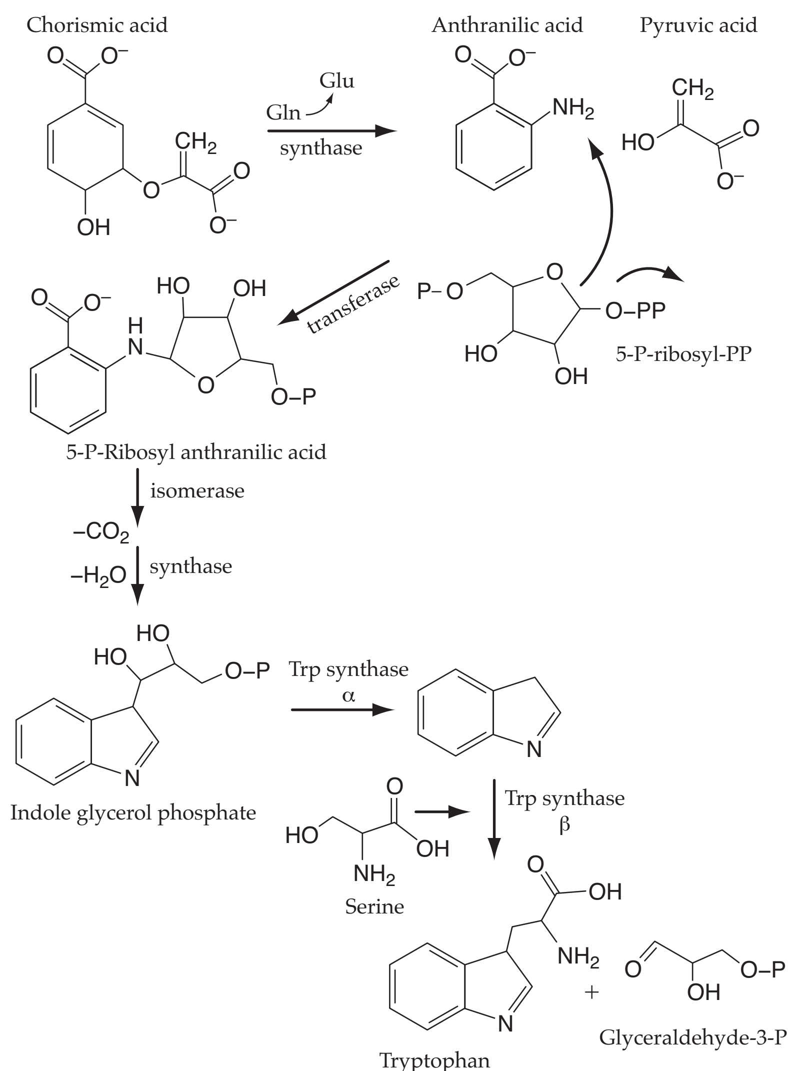

FIGURE 8.8 Synthesis of tryptophan. Anthranilate synthase binds chorismic acid and catalyzes removal of the pyruvyl moiety followed by transfer of an amino group from glutamine to the ring to form anthranilic acid. The sugar, 5-phosphate-ribosyl-diphosphate, is coupled to the amino group in anthranilate by an *N*-glycosidic bond and pyrophosphate is released. The 5-phospho-ribosyl moiety then undergoes isomerization to a ketose. The ring of the intermediate is decarboxylated, and a third enzyme catalyzes a ring closure of the ketose to generate the five-membered ring in indole glycerol-3-phosphate. This intermediate is the substrate for the α subunit of $\alpha_2\beta_2$ tryptophan synthase. Binding of indole glycerol-phosphate to the α subunit closes this subunit active site, and the enzyme cleaves the glyceryl-phosphate moiety from the indole ring. The products are not released from the enzyme. The indole intermediate is transported through a channel in the enzyme to the β subunit. Serine also binds to the β subunit, and binding closes this second active site. The hydroxyl group is removed from serine, and the remaining three-carbon fragment is coupled to the indole ring to generate tryptophan. Opening of the active sites on both subunits releases tryptophan from the β subunit and glyceraldehyde-3-phosphate from the α-subunit site.

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8.3 Phenylpropanoid Pathway

Many complex plant products are synthesized from the aromatic amino acids, including both primary and secondary metabolites (natural products). Phenylalanine occupies a particularly prominent position in plant metabolism as it is the starting material for the synthesis of a large number of aromatic compounds. These metabolites are referred to as phenylpropanoids and include primary products such as the monolignol precursors of lignin and a large number of species-specific natural products. Lignin is a major component of secondary cell walls and the second most abundant polymer in the biosphere after cellulose. Its synthesis is vital to the survival of all terrestrial plants. Lignin is a polymer of phenylpropanoid precursors that are exported to the cell wall matrix of mature plant tissues. In the wall the precursors are polymerized and form crosslinks to components of the primary cell wall. Phenylpropanoids are also the precursors for phytoalexins in some plants and volatiles such as eugenol found in cloves. Another major role of phenylpropanoids is in the production of polyketides, including flavonoids, which are found in most plants and enable them to interact and cope with environmental factors. All this complex variety of end products starts with a phenylalanine precursor.

Synthesis of *Trans*-Cinnamic Acid

The synthesis of phenylpropanoids starts with the removal of the amino group of phenylalanine to produce *trans*-cinnamic acid. The enzyme that catalyzes this initial step is phenylalanine ammonia lyase (PAL). PAL is found in angiosperms and gymnosperms and also in some mosses, algae, fungi, and bacteria. Several PAL isozymes occur in angiosperms. The plant enzymes are found in the cytoplasm, most likely associated with the outer face of the endoplasmic reticulum. PAL from various plant sources is a tetramer with a mass of 225 to 330 kDa. In dicotyledonous plants, PAL is specific for phenylalanine. Some dicots also have another enzyme that deaminates tyrosine (tyrosine ammonia lyase). In monocots, PAL accepts both phenylalanine and tyrosine as substrates, although the catalytic efficiency is lower for tyrosine.

Plant PAL belongs to the amino acid–ammonia–lyase family of enzymes. The plant enzyme is homologous to the bacterial histidine ammonia lyase (HAL) and has the same mechanism of action. The crystal structure of PAL from parsley (*Petroselinum crispum*) has been determined. The protein is mainly composed of α-helices, and its overall tertiary structure is very similar to the PAL from *Rhodosporidium toruloides* and histidine ammonia lyase from the bacterium, *Pseudomonas syringae*. All these enzymes are unique in that they generate their own cofactor at the active site by cyclization and dehydration of an alanine-serine-glycine sequence to generate 4-methylidene-imidazole-5-one (MIO) (**FIGURE 8.9**). The methylidene group of the MIO cofactor binds the amino group of the substrate, phenylalanine, and weakens the C–H bond on the β-carbon of the substrate

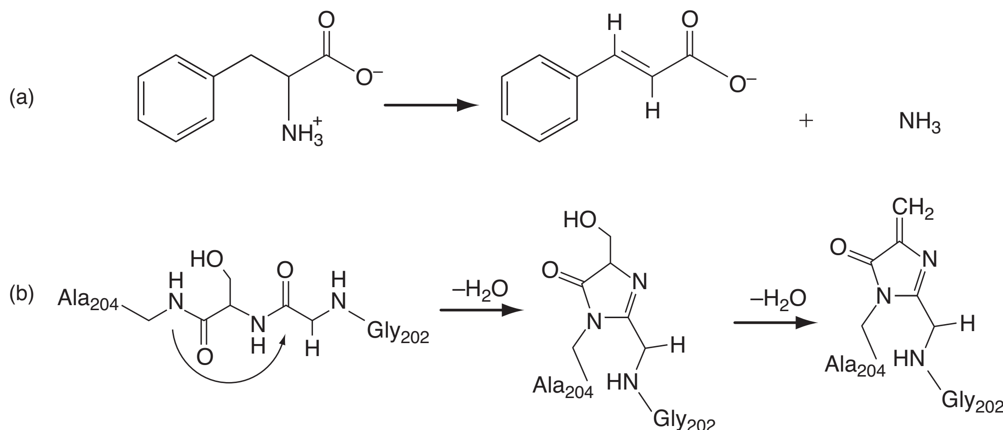

FIGURE 8.9 Activity of phenylalanine ammonia lyase (PAL). (a) PAL catalyzes the removal of the amino group from the α-carbon and a hydrogen from the β-carbon of phenylalanine to produce *trans*-cinnamic acid and ammonia. (b) The enzyme generates a unique cofactor at its active site by cyclization of three residues, alanine-204, serine-203, and glycine-202 (residue numbering is from the bacterial enzyme), followed by a dehydration. The amino group in the peptide bond between ala-204 and ser-203 attacks the carbonyl carbon in the peptide bond between ser-203 and gly-202. Subsequent removal of two H_2O molecules generates the 4-methylidene-imidazole-5-one (MIO) cofactor that binds the substrate, phenylalanine, and facilitates removal of the amino group to generate NH_3 (shown in Figure 8.10).

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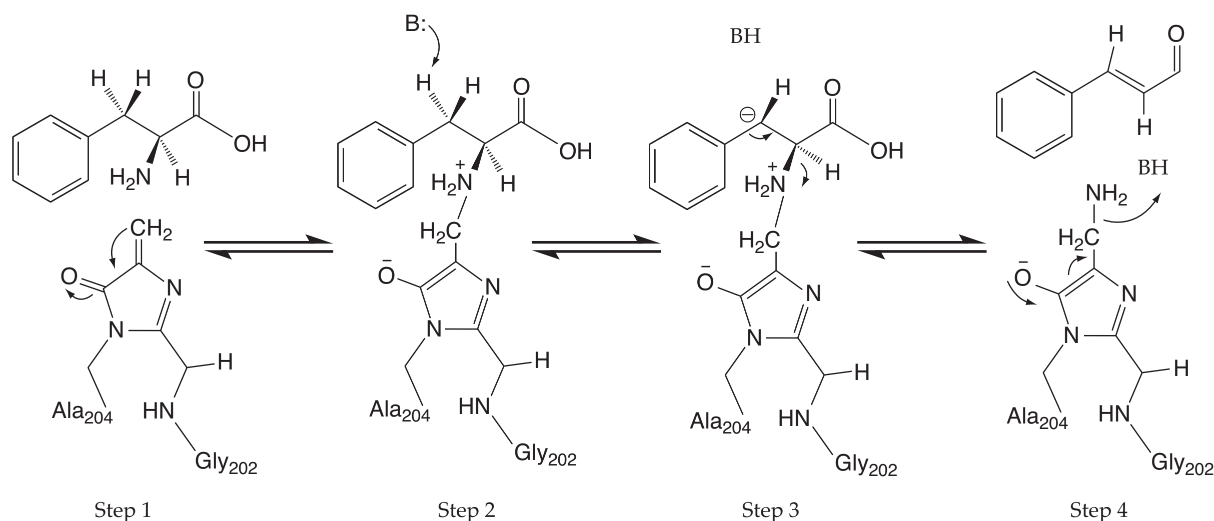

FIGURE 8.10 Mechanism of action of phenylalanine ammonia lyase (PAL). This enzyme is the gateway to the phenylpropanoid pathway, and its mechanism has been thoroughly investigated. Step 1: The methylidene carbon of the MIO cofactor (Figure 8.9B) in PAL binds to the amino group of phenylalanine. Step 2: A basic group in the enzyme, B, stereospecifically removes one of the protons from the β-carbon of phenylalanine. Step 3: Rearrangement of the resulting anion generates a double bond in the substrate and facilitates cleavage of the bond between the α-carbon of phenylalanine and its amino group. Step 4: *Trans*-cinnamic acid is released from the enzyme. The protonated BH residue in the active site donates its H^+ to the aminated cofactor and facilitates removal of the NH_3 and regeneration of MIO. (*Mechanism adapted from*: Calabrese, J. et al., "Crystal Structure of Phenylalanine Ammonia Lyase: Multiple Helix Dipoles Implicated in Catalysis," *Biochemistry* 43: [2004] 11403–416.)

(**FIGURE 8.10**). A proton is removed from this β-carbon with the help of a basic group in the active site, most likely a histidine residue. Breaking of the very stable C–H bond in this reaction is facilitated by the electrophilic environment around the active site created by the aligned helix dipoles of the enzyme. Removal of the proton generates an anion that rearranges and results in the cleavage of the C–N bond between phenylalanine and the MIO cofactor. The product, *trans*-cinnamic acid, is released from the active site. The cofactor is regenerated by H^+ donation from an acidic residue in the enzyme and ammonia is produced. *Trans*-cinnamic acid is the precursor for most of the phenylpropanoids, including the monolignols, used to synthesize lignin.

Lignin Synthesis

Lignin is a highly irregular polymer formed from aromatic alcohols referred to as monolignols. The major monolignols are coumaroyl, coniferyl, and sinapyl alcohols. These precursors are synthesized from *trans*-cinnamic acid by enzymes associated with the endoplasmic reticulum. The monolignols are exported to the cell wall, and polymerization of the monolignols then occurs outside the cell membrane.

Synthesis of *p*-Coumaroyl Alcohol The aromatic ring of *trans*-cinnamic acid can be hydroxylated by a specific cytochrome P450 monooxygenase. The monooxygenases in this pathway are often referred to as hydroxylases. The hydroxyl (OH) group is added at the *para*-position with respect to the side chain to produce 4-coumaric acid (*p*-coumaric acid). Coumaric acid can also be produced in some plants by direct deamination of tyrosine in a TAL-catalyzed reaction (**FIGURE 8.11**).

Coumaric acid can be ligated to coenzyme A by a ligase; this reaction requires two equivalents of ATP to activate coumaric acid and catalyze the formation of a thioester bond. Coumaroyl-CoA is a major branch point in the synthesis of monolignols and other phenylpropanoids. There is experimental evidence that all the monolignols are present as CoA esters until a final reduction to the alcohols, but some of the modifying enzymes can use the free acids or other derivatives as substrates. In addition, recent analyses have also shown that monolignols are present as complexes with shikimic or quinic acids, which are intermediates in the shikimic acid pathway (**FIGURE 8.12**). Because these acids are synthesized in plastids, it is assumed that they must be specifically transported to the cytoplasm to serve as monolignol carriers.

Para-coumaroyl-CoA, or other conjugate or the free acid, undergoes reduction to coumaroyl alcohol by two reductases that use NADPH as a cofactor. The mechanism of action of these two enzymes is slightly different. The first enzyme, coumaroyl-CoA reductase (CCR),

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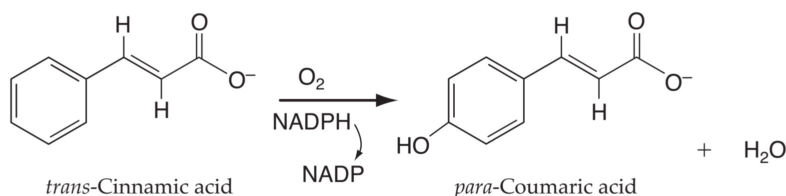

FIGURE 8.11 Synthesis of *p*-coumaric acid. A hydroxyl (OH) group is added to *trans*-cinnamic acid by a cytochrome P450 monooxygenase (hydroxylase). The enzyme adds the OH-group at the *para* position with respect to the side chain. Coumaric acid can also be synthesized by direct deamination of tyrosine catalyzed by tyrosine ammonia lyase (TAL) in some plants.

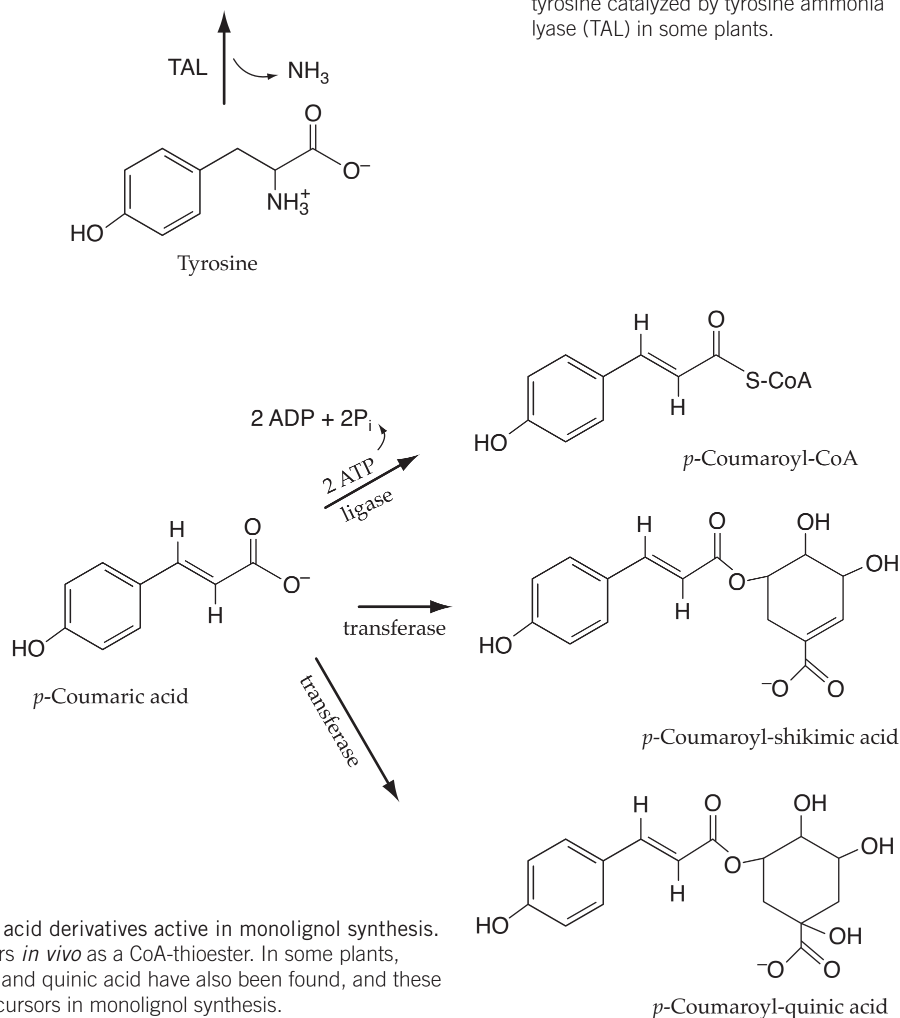

FIGURE 8.12 *Para*-coumaric acid derivatives active in monolignol synthesis. Coumaric acid generally occurs *in vivo* as a CoA-thioester. In some plants, conjugates with shikimic acid and quinic acid have also been found, and these may function as alternate precursors in monolignol synthesis.

reduces the acid to the aldehyde. It is referred to as a type B reductase because it transfers the HB hydride from NADPH (**FIGURE 8.13**). The second reductase, coumaroyl aldehyde dehydrogenase (CAD), is a type A reductase. It transfers the HA hydride from the cofactor and reduces the aldehyde to an alcohol. These reductases are highly specific and cannot substitute for each other. This has been demonstrated in genetically altered plants designed to generate different pools of monolignols.

Coumaroyl alcohol is one of the monolignol precursors. It can be polymerized into a complex referred to as *p*-hydroxyphenyl lignin, or H-lignin. H-lignin is common in some monocots, but in most plants it is often a minor component found in complexes with other types of lignin.

Synthesis of Coniferyl and Sinapyl Alcohols Coumaric acid, either as the free acid or a derivative, is hydroxylated by a second monooxygenase to produce the dihydroxy derivative, caffeic acid. This second OH group in the aromatic ring is then methylated by an O-methyl transferase that requires S-adenosyl methionine (SAM) as the methyl donor. Monohydroxy substituted rings are usually not good substrates for the methyl transferases, but they can bind a number of different hydroxylated substrates. The methylated caffeic acid is ferulic acid (**FIGURE 8.14**). Ferulic acid is then

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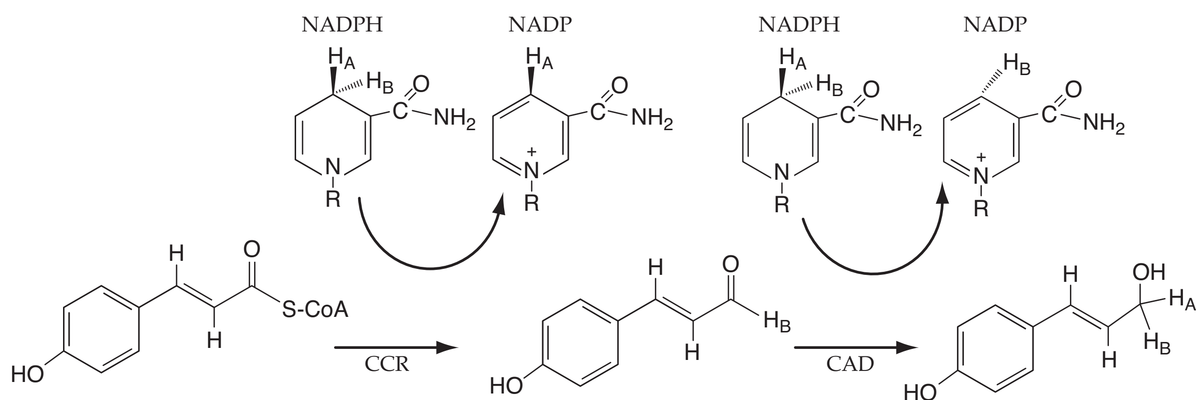

FIGURE 8.13 Reductases that reduce *p*-coumaroyl-CoA and other monolignol precursors. The first enzyme, *p*-coumaroyl-CoA reductase (CCR, also called cinnamate-CoA reductase), transfers the HB hydride from NADPH and reduces the thioester to the aldehyde. It is referred to as a type B reductase. The second enzyme, coumaroyl aldehyde dehydrogenase (CAD), transfers the HA hydride from the cofactor to produce coumaroyl alcohol; it is a type A reductase. Both CCR and CAD can also catalyze reduction of other monolignol precursors; NADPH is the reducing agent in both reactions.

p-Coumaroyl-CoA

NADPH → NADP, O_2

Caffeoyl-CoA + H_2O

S-Adenosyl methionine (SAM)

O-Methyl transferase

S-Adenosyl homocysteine (SAH)

Feruloyl-CoA

NADPH → NADP, CCR, CoASH

Coniferyl aldehyde

NADPH → NADP, CAD

Coniferyl alcohol

Guaiacyl lignin (G-lignin)

FIGURE 8.14 Synthesis of coniferyl alcohol. Coumaroyl-CoA is hydroxylated by a specific cytochrome P450 monooxygenase at the *meta* position (with respect to the side chain) to generate caffeic acid or a caffeoyl conjugate. A caffeoyl O-methyl transferase then adds a methyl group to this *meta*-hydroxyl group to produce ferulic acid. The methyl donor is S-adenosyl methionine (SAM). Removal of the methyl group from SAM yields S-adenosyl homocysteine (SAH), which can be remethylated. Ferulic acid and its conjugates are reduced by the *p*-coumaroyl-CoA reductase (CCR) and coumaroyl aldehyde dehydrogenase (CAD) reductases described in Figure 8.13 to yield coniferyl alcohol. This product is a lignin precursor that can be polymerized to guaiacyl or G-lignin, which is the major lignin type found in gymnosperms, especially conifers.

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reduced to the alcohol by the same redox enzymes, CCR and CAD, described in Figure 8.13 to yield coniferyl alcohol. This alcohol is the major component of guaiacyl lignin (G-lignin).

Another cytochrome P450 monooxygenase introduces a third hydroxyl group into the aromatic ring to produce 5-hydroxy ferulic acid. This third hydroxyl group is methylated by a SAM-dependent transferase, as shown in **FIGURE 8.15**, to produce sinapic acid. Sinapic acid is then reduced to sinapyl alcohol, a major monolignol made in dicotyledonous angiosperms and the precursor of syringyl lignin (S-lignin).

Polymerization of Monolignols

The monolignol precursors, coumaroyl, coniferyl, and sinapyl alcohols, are exported to the exterior of the cell. They may be packaged into Golgi vesicles or transported directly. In some mature plant tissues monolignols are found as glycosylated derivatives, presumably for storage until secondary wall synthesis is induced. Polymerization of the monolignols occurs outside the cell membrane, between it and the primary cell wall. The polymerization process is nonenzymatic but is initiated by an enzymatic free radical formation. Two types of oxidases, peroxidases and laccases, are found

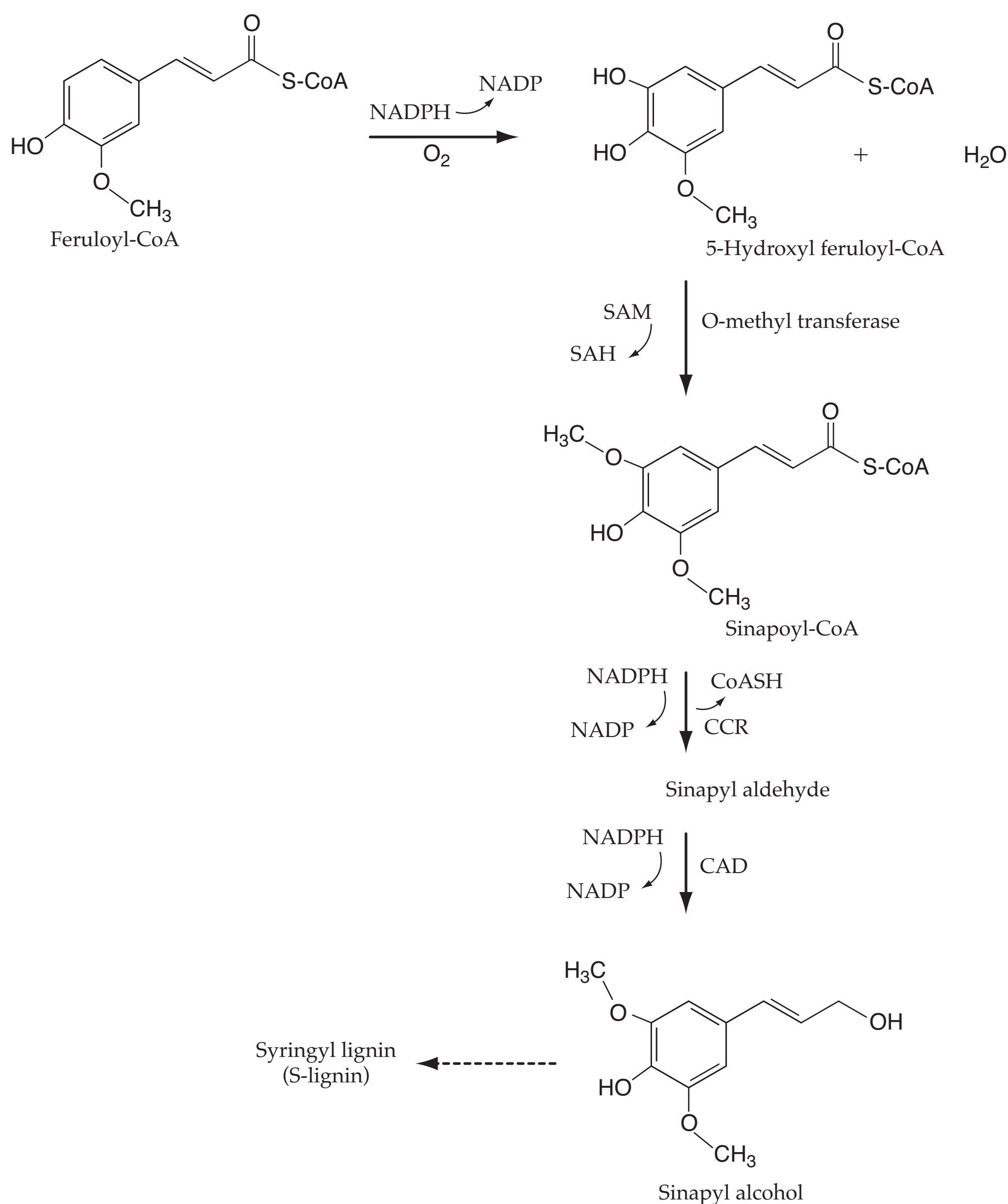

FIGURE 8.15 Synthesis of sinapyl alcohol. Ferulic acid, or a derivative, is hydroxylated by a cytochrome P450 monooxygenase to 5-hydroxy ferulic acid. An O-methyl transferase adds a methyl group to this third hydroxyl group to produce sinapic acid, which is reduced to the alcohol by *p*-coumaroyl-CoA reductase (CCR) and coumaroyl aldehyde dehydrogenase (CAD). Sinapyl alcohol is a primary monolignol that is polymerized into syringyl or S-lignin, a major component of secondary cell walls in dicotyledonous plants.

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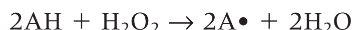
in the cell wall that can act as free radical initiators. Peroxidases are heme-Fe containing enzymes that use a variety of substrates and hydrogen peroxide to generate a radical:



$$2AH + H_2O_2 \rightarrow 2A\bullet + 2H_2O$$

Plants contain a number of isoforms of these enzymes; the peroxidases in the cell wall are designated class III peroxidases. They can generate reactive oxygen species (ROS, see Appendix 5) associated with the wound response. Peroxidases are also capable of generating monolignol radicals. The source of the hydrogen peroxide in the cell wall is unknown but could presumably be generated by the peroxidase itself from molecular oxygen.

The second type of radical initiator, the laccases, are Cu^+-containing proteins belonging to the phenol oxidase enzyme family. They generate radicals from molecular oxygen:

$$4AH + O_2 \rightarrow 4A\bullet + 2H_2O$$

An unpaired electron that is introduced into a monolignol can be delocalized over the aromatic ring and side chain and subsequently couple with another radical to form a bond in a number of different linkages. Some of the possible linkages between monolignols are shown in **FIGURE 8.16**. A similar random radical mechanism links lignin to carbohydrates in the matrix of the primary cell wall (see Chapter 4).

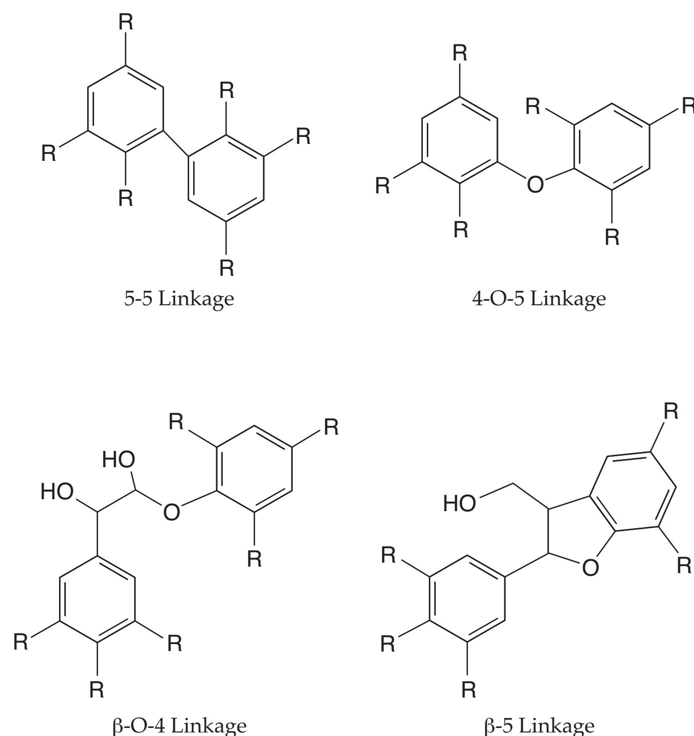


FIGURE 8.16 Some common linkages found in lignin. The types of linkages found in lignin depend on the pool of monolignols that are synthesized and exported from the cell. In conifers, coniferyl alcohol is the most abundant precursor, and the resulting G-lignin contains mainly C–C bonds such as the 5-5 linkage shown. Dicotyledonous angiosperms produce mainly sinapyl alcohol precursors and the resulting S-lignin contains more ether linkages such as the 4-O-5 and β-O-4 linkages. Because most plants produce some of each type of monolignol, all the above linkages and many mixed linkages such as the β-5 configuration with both C–C and C–O bonds are possible.

Unlike the carbohydrates of the primary cell wall, lignin is a relatively inflexible polymer. It makes cell walls rigid and provides support for plant stems and vascular tissues. It is especially important in xylem vessels where it maintains the vessel structure under negative water pressure. Lignin also provides a barrier to pathogen invasion. The lignin structure with its random chemical bonds is highly resistant to both enzymatic and chemical degradation. Only a few organisms, mostly fungi, can degrade lignin; they use a free radical mechanism to generate random breaks in the polymer, essentially reversing the polymerization process.

Genetic Engineering of Lignin

The properties of lignin in plants depend on the concentration of monolignols exported to the wall, which determines the different types, H, G, or S. Gymnosperm wood is mainly G-lignin; the monolignol pool contains mostly coniferyl alcohol with smaller amounts of sinapyl alcohol. This pool gives rise to a type of lignin with mainly C–C linkages that are more stable than the ether linkages that result from a higher concentration of sinapyl alcohol precursors (**FIGURE 8.17**). Plants containing a high content of G-lignin are more difficult for herbivores, such as domestic cattle and sheep, to digest. G-lignin also requires harsher chemical treatment in the pulping process to make paper and presents a barrier to the production of ethanol-based biofuels.

Many research groups have attempted to genetically alter the composition of lignin with the goal of producing a product that is easier to degrade, either enzymatically or chemically. Examples of these manipulations include a gene knock-out or down-regulation of the enzyme that methylates caffeoyl-CoA (CCoAOMT). This should result in a pool of monolignols with a higher concentration of coumaroyl alcohol (use **FIGURE 8.18** as a guide). The engineered lignin that results from this manipulation should have less of both the G- and S-lignin components

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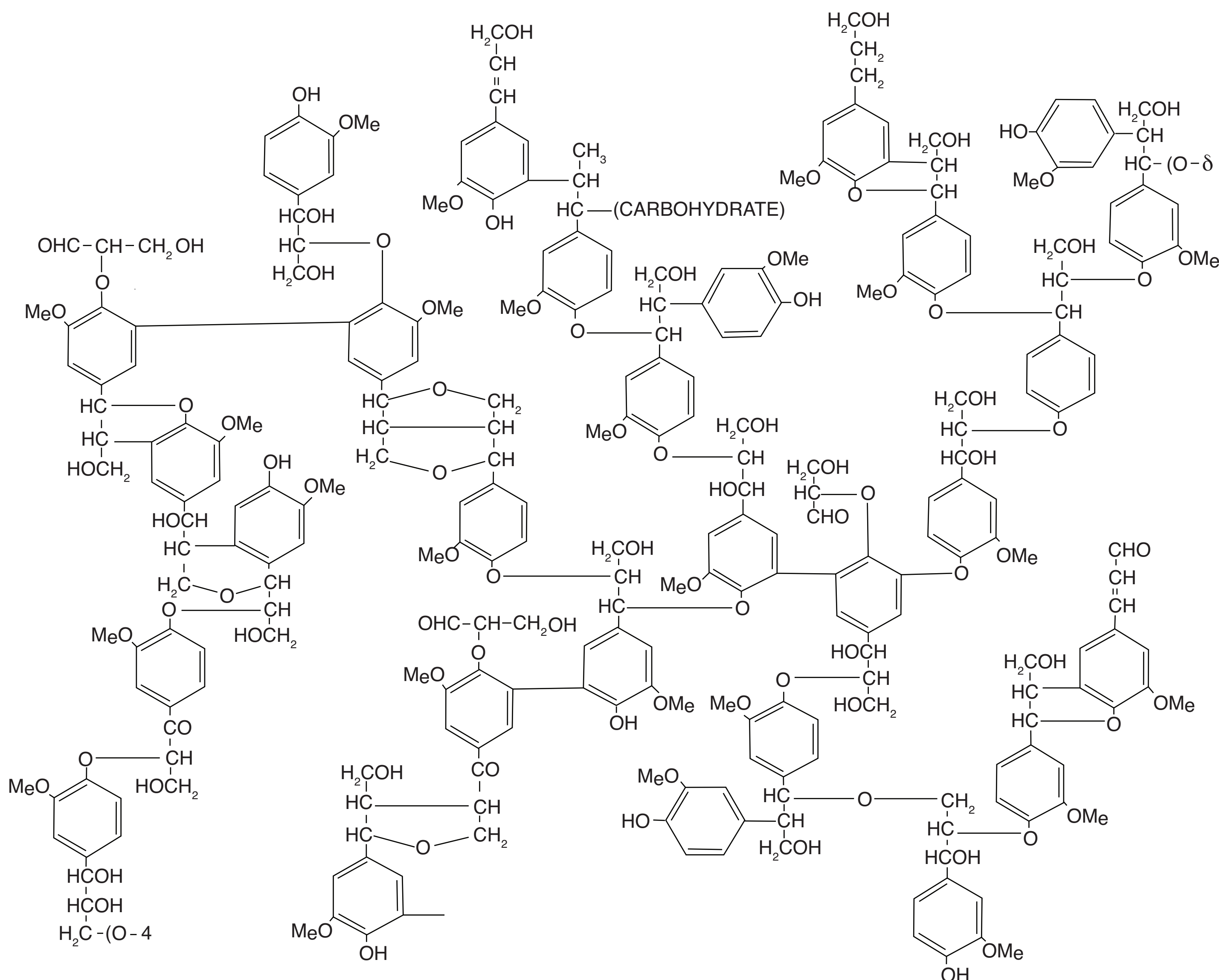

FIGURE 8.17 Model of linkages found in conifer lignin. These are some of the major linkages that can be found in G-lignin formed predominantly from coniferyl alcohol. Both C–C and C–O bonds are formed, but the C–C linkages are more common in this type of secondary cell wall polymer. (*Reproduced from*: M. Baucher, B. Monties, M. Van Montagu, et al. *Crit. Rev. in Plant Sci*. 17 [1998]: 125–197, Figure 7. Courtesy of Marc Van Montagu, Ghent University.)

and therefore fewer recalcitrant C–C bonds. The lignin in transgenic tobacco and poplar plants, however, actually had a higher content of 5-hydroxyguaiacyl units (i.e., more coumaroyl alcohol was incorporated into the polymer), but additional 5-hydroxyferulic alcohol was also found. Apparently, the other O-methyl transferases can adequately substitute for CCoAOMT in these plants, and the resulting modified lignin still contains a high proportion of linkages that are resistant to degradation.

Naturally occurring mutations in the monolignol enzyme genes were found in maize (*Zea mays*) and were designated *bm1,2,3,4* (*brown mid-rib*). The *bm3* mutant was found to have reduced O-methyl transferase (COMT) activity (see Figure 8.18). The mutant plants contain less S-lignin in their walls as expected but again compensated with a higher content of hydroxyferulic alcohol. The mutant maize leaves and stalks are more digestible by ruminants. Grain yield is lower in mutant plants, however, and they are more susceptible to lodging (i.e., stems break easily in the wind).

In the model plant, *Arabidopsis*, mutants with knock-out mutations in the gene for ferulic acid monooxygenase (F5H) produce no S-lignin. Conversely, overexpression of the gene for this monooxygenase leads to production of cell walls that are predominantly S-lignin, suggesting that manipulation of the expression of the ferulate-5-hydroxylase gene

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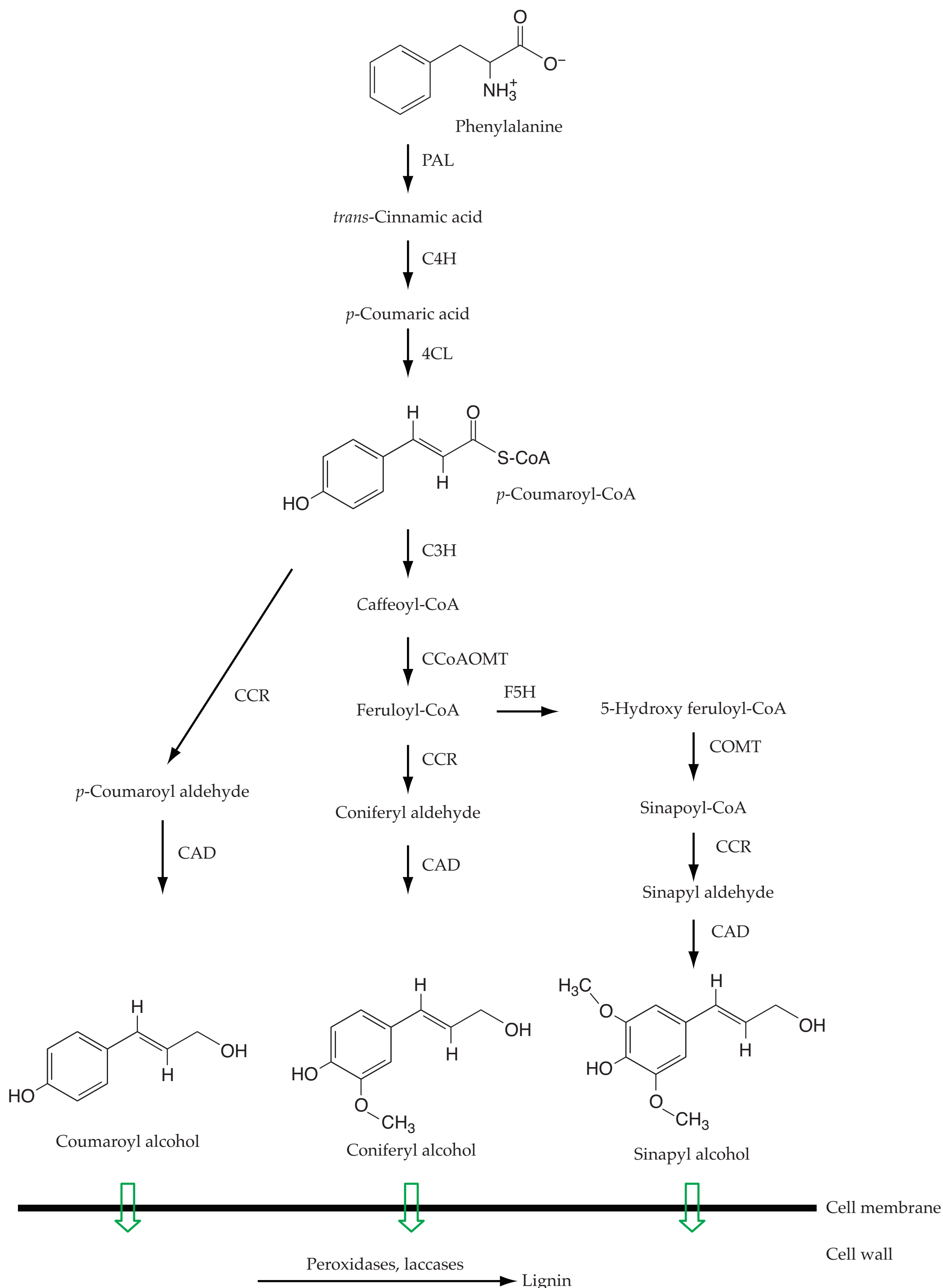

FIGURE 8.18 Simplified map of monolignol and lignin synthesis in vascular plants. The most direct route to the three major monolignols synthesized from *p*-coumaric acid and some of the enzymes required are shown. In most plants, alternate reactions/enzymes are present, providing additional routes to the major monolignols and other compounds that may be found in lignin. The *in vivo* pathway for lignin synthesis is more grid-like than this simple linear diagram. The mechanism by which monolignols are transported across the cell membrane is unknown. CAD, coumaroyl alcohol dehydrogenase; CCoAOMT, caffeoyl-CoA-O-methyl transferase; C3H, *p*-coumarate-3-hydroxylase (monooxygenase); C4H, coumaroyl-4-hydroxylase; CCR, coumaroyl-CoA reductase; 4CL, 4-coumarate CoA ligase; COMT, caffeic acid/5-hydroxy ferulic acid O-methyltransferase; F5H, ferulate-5-hydroxylase; PAL, phenylalanine ammonia lyase.

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may be a key step in increasing the digestibility of forage crops.

The results in any one plant system, however, may not be applicable to other species. Down-regulation of the gene for 4-coumarate CoA ligase (4CL) in tobacco produced plants with less overall lignin in the cell walls and a polymer with a higher content of coumaroyl alcohol. Conversely, in *Arabidopsis* the total lignin content in 4-coumarate CoA ligase mutant plants was reduced but the walls still contained high levels of S-lignin. This suggests that alternate pathways for monolignol synthesis are present in some species and can compensate for alterations in the main sequence of monolignol production, but these alternatives may be species specific. The striking results in poplar (*Populus tremuloides*) illustrate the flexibility of plants in producing lignins. Down-regulation of the aldehyde reductase (CAD) synthesis results in plants with a higher content of aldehydes in their cell walls. Aldehyde incorporation produces a pronounced red coloration in the vascular tissues of the woody stems. Although genomics and genetic engineering techniques have greatly increased our knowledge of lignin synthesis, application of these techniques to manipulate the quantity and properties of lignin is still problematic because of our overall lack of understanding of how cell walls are made. Methods for analyzing lignin and linkages, such as nuclear magnetic resonance (NMR) spectroscopy, are a valuable asset to lignin engineers and are currently used to provide a more directed path to lignin manipulation.

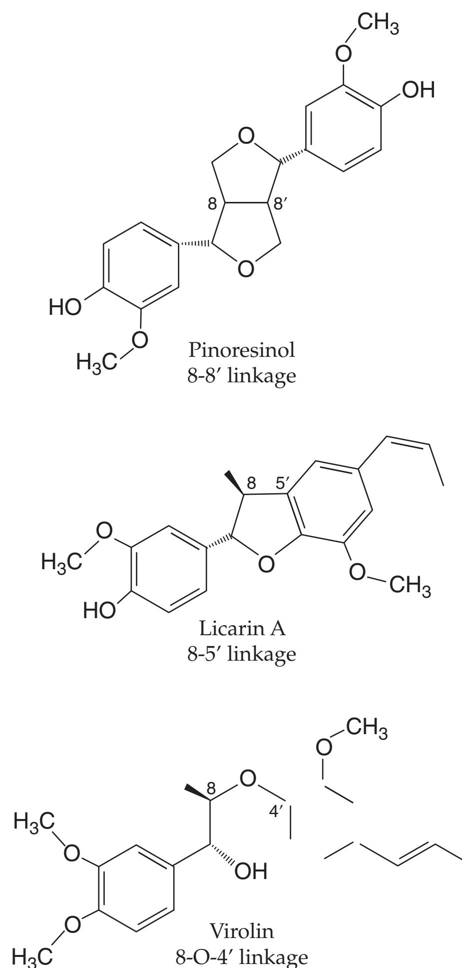

FIGURE 8.19 Structures of some common lignans. Lignans are dimers of monolignols with precise linkages formed between the precursors. They are synthesized by free radical–initiated bond formation guided by dirigent proteins. The dilignol bonds in these compounds correspond to linkages found in lignin (see Figure 8.16).

8.4 Natural Products Derived from the Phenylpropanoid Pathway

Natural Products from Monolignols

Lignans Lignans are dimeric phenylpropanoids that are synthesized from monolignols, mainly coniferyl alcohol. They are found in the seed coats, flowers, and epidermal layers of stems and leaves where they serve to defend plant tissues against pathogens. They are also precursors for a variety of complex natural products. Lignans are synthesized by a free radical–coupling mechanism, but the linkages are specific rather than random as in lignin. The structures of some common lignans are shown in **FIGURE 8.19**. The monolignol substrates are held in position by proteins called dirigents (guide proteins). These proteins have no enzymatic activity; they serve to bind the lignols and guide the radical-mediated bond formation (**FIGURE 8.20**). As in the case of lignin synthesis, the free radical initiator is a peroxidase.

Lignan dirigent proteins are found in the cytoplasm, but similar dirigents have been localized in the cell walls of some plants. It has been proposed that these extracellular dirigent proteins may be active in lignin formation. The binding of monolignols to dirigents with different sites could account for the regular bonding patterns found in some lignin polymers. Although a large number of different dirigent proteins and their genes have been predicted from genomic analyses, an even larger number would presumably be required to guide total lignin synthesis. The dirigent proteins in cell walls may determine the bonding in the initial products of polymerization to which additional monolignols are then added in a random, radical-coupled process.

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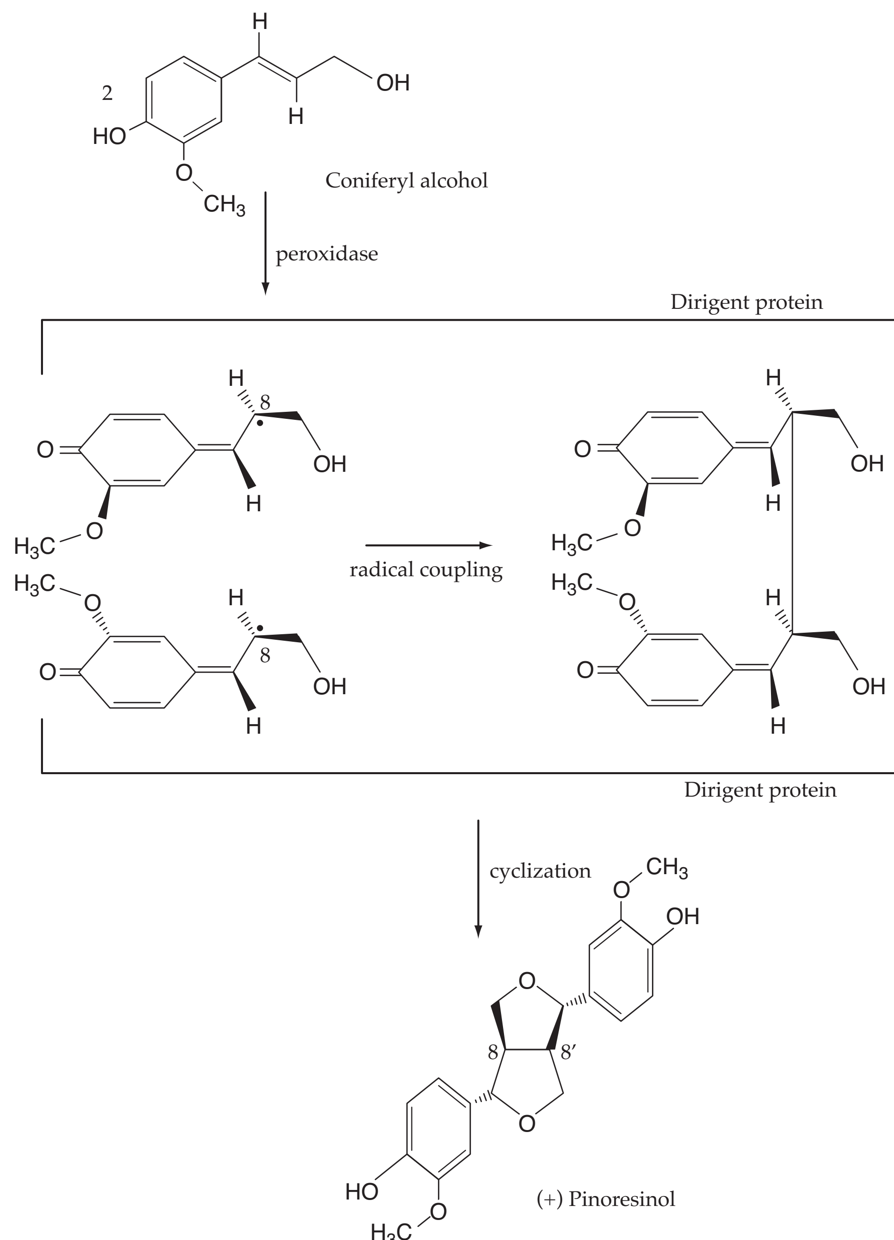

FIGURE 8.20 Dirigent proteins guide the synthesis of lignans. The dirigent proteins are represented by brackets. The proteins bind two molecules of coniferyl alcohol in this example and hold them in a defined position. A radical is generated by a cytoplasmic peroxidase. Because of binding to the dirigents, radical coupling occurs only between the 8-8′-positions. The product is the lignan, pinoresinol, a compound that is common in the Oleaceae (olive family).

In some plants, lignans are modified to produce species-specific natural products. Some of these modified lignans are useful to humans as anticancer agents and nutritional supplements. The structures of three examples are shown in **FIGURE 8.21**. Sesamin is an antioxidant found in the seed oil of the sesame plant (*Sesamum indicum*). Gomisin A, from *Schisandra chinensis*, a tropical vine from Asia, is used in traditional herbal medicines as a treatment for liver disorders. Podophyllotoxin from May apple (*Podophyllum peltatum*) inhibits cell division by binding to tubulin and preventing the formation of the spindle apparatus. It is, therefore, a potential anticancer agent but is too toxic to be taken internally. It is used topically to treat some skin cancers. A synthetic derivative, etoposide, is less toxic and can be taken internally to treat certain types of lung and testicular cancers. Etoposide acts by inhibiting topoisomerase activity and DNA synthesis rather than binding to tubulin.

Volatiles Many plants produce volatile compounds from monolignols. Volatiles in flowers and fruits are attractive to pollinators and her-

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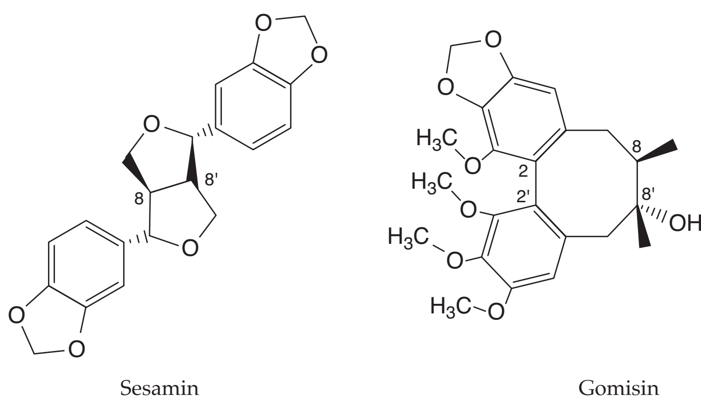
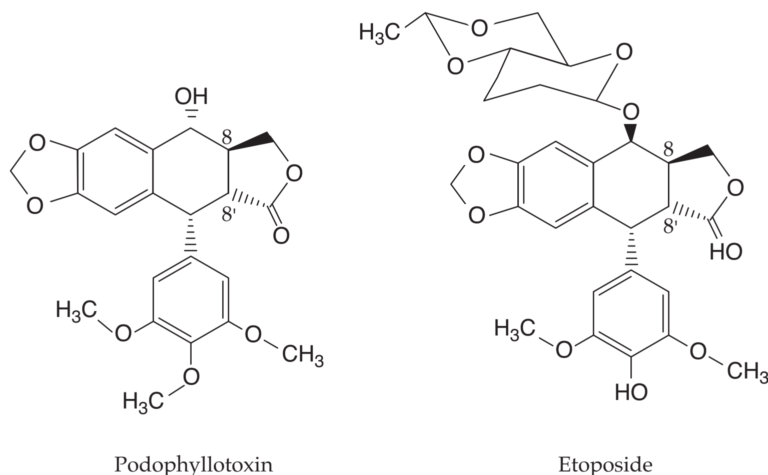


FIGURE 8.21 Structures of modified lignans that are useful to humans. Sesamin is an antioxidant found in sesame seed oil that is marketed as a neutraceutical. Gomisin-A has an unusual eight-membered ring. It is the active ingredient in some traditional herbal medicines used to treat liver disorders. Podophyllotoxin inhibits tubulin polymerization and prevents cell division. Although podophyllotoxin is too toxic for therapeutic use, it is the natural model for etoposide, a synthetic anticancer drug.



bivores, while volatile compounds in leaves and stems repel insects or attract insect predators. A common pathway for the synthesis of some volatiles is shown in **FIGURE 8.22**. The alcohol group of monolignols such as coniferyl alcohol can be removed by a reductase to generate a resonance-stabilized carbocation intermediate. Reduction of the allylic cation yields eugenol, the aromatic compound found in cloves (*Syzgium aromaticum*), which are used by humans as both a flavoring agent and an analgesic (pain reliever). Similarly, reduction of the corresponding propenyl cation and dehydration produces anethole, a volatile found in many plants such

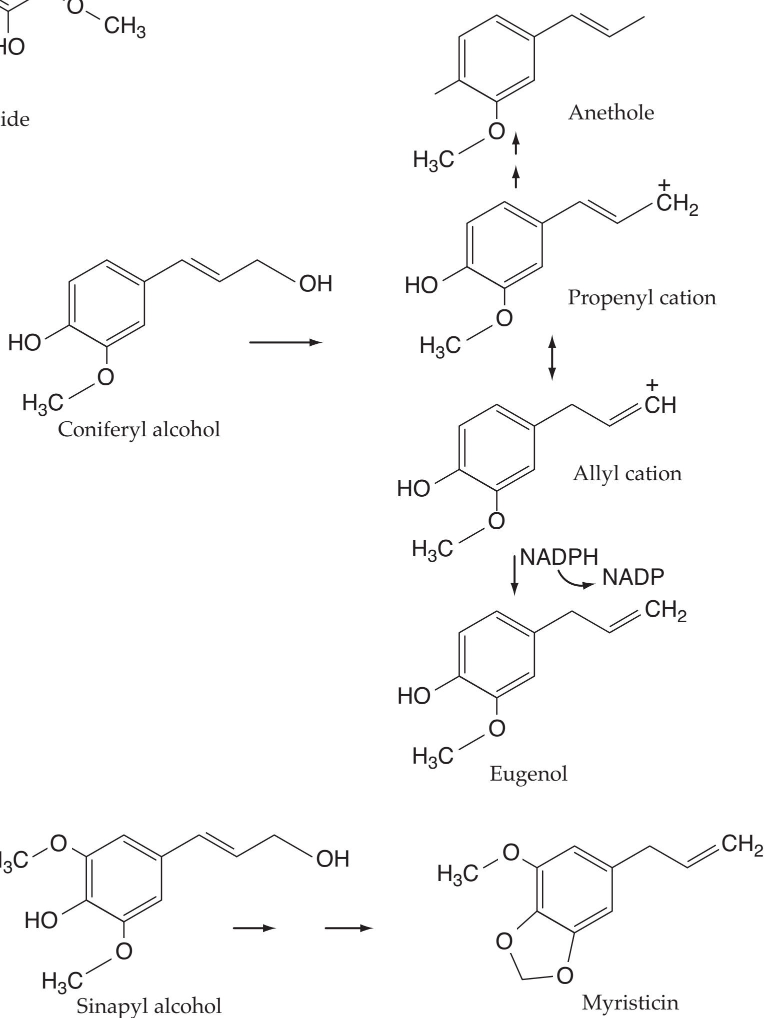


FIGURE 8.22 Examples of volatile natural products produced from monolignols. Removal of the hydroxyl group from coniferyl alcohol generates two resonance- stabilized carbocations. Reduction of the allylic cation generates the methylene group in eugenol, the volatile compound in cloves. Reduction of the propenyl cation and subsequent dehydration generates anethole, the volatile found in many plants such as fennel. Removal of the hydroxyl group of sinapyl alcohol and reduction of the resulting allylic cation to a methylene group initiates the formation of myristicin, which also requires ring closure to form the methylene dioxy ring. Myristicin is the volatile that adds to the fragrance of nutmeg seeds.

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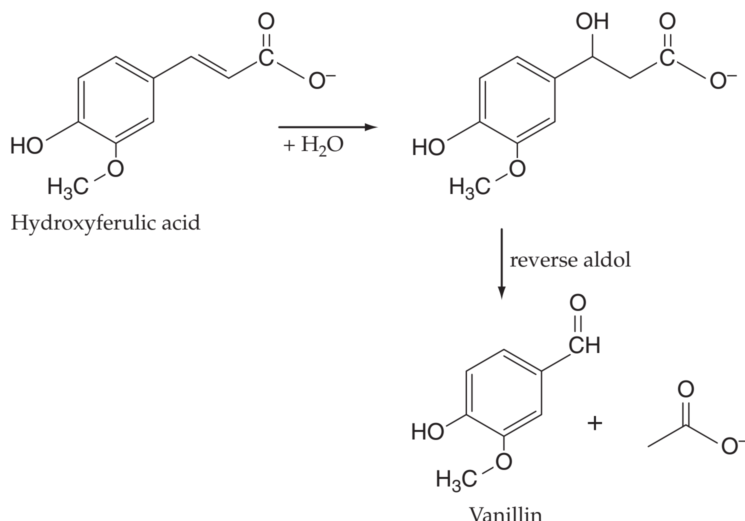

FIGURE 8.23 Synthesis of vanillin. Vanillin is produced by a fermentation process that alters ferulic acid derivatives found in secondary cell walls. Hydration of the side chain of hydroxyferulic acid is followed by a reverse aldol reaction to generate the aldehyde, vanillin.

as fennel (*Foeniculum vulgare*). If the starting material is sinapyl alcohol, a similar reduction and subsequent formation of a methylene dioxy ring yields myristicin (Figure 8.22), the volatile compound found in nutmeg (*Myristica fragrans*).

Vanillin is a natural product derived from hydroxyferulic acid. In the pathway illustrated in **FIGURE 8.23**, the double bond in hydroxyferulic acid is hydrated followed by a reverse aldol reaction that cleaves the side chain to yield vanillin. In some plants an alternate route uses the CoA ester of ferulic acid as a precursor. Commercial vanilla extract from the pods of the vanilla orchid (*Vanilla planiflora*) is produced after a long fermentation period. The pods are then dried and extracted with ethanol. Real vanilla extract contains vanillin and a number of additional compounds. Imitation vanilla extracts can be produced by fermentation of the ferulic acid found in some woody stems.

Salicylic Acid The plant hormone, salicylic acid, can be synthesized by a variety of pathways. It is derived in some plants from coumaroyl-CoA. In bacteria and other plants, such as *Arabidopsis*, salicylic acid can be produced from chorismic acid, an intermediate in the shikimic acid pathway (Figures 8.1 and 8.7). Salicylic acid is a hormone produced after pathogen invasion. It is transported to undamaged plant tissues where it mediates systemic acquired resistance (SAR). It is also the active ingredient in aspirin (acetyl salicylic acid), although the modern commercial product is made by chemical synthesis.

8.5 Synthesis and Properties of Polyketides

As described in the previous section, the phenylpropanoid pathway furnishes monolignols that are essential for lignin synthesis and, therefore, can be considered part of the primary metabolism of plants. This same pathway also provides precursors for many different aromatic compounds that are unique to a particular plant, such as eugenol and myristicin (Figure 8.22), and are generally regarded as secondary metabolites or natural products. In addition, coumaroyl-CoA from the phenylpropanoid pathway is the starting material for the synthesis of polyketides such as the flavonoids, which have traditionally been considered natural products.

Many flavonoids are unique to a particular family of plants. Both their synthesis in a specific plant and molecular details have been used to construct biochemically based phylogenies. Most land plants and many aquatic species, however, contain some class of flavonoids that function to protect the organisms against UV radiation, free-radical damage, pathogens, and herbivores or to attract pollinators, mycorrhizal fungi, and symbiotic microorganisms. Given their essential roles in enabling plants to interact with both biotic and abiotic factors in their environment, flavonoids and other polyketides are definitely of primary importance in plant metabolism.

Synthesis of Chalcones

Coumaroyl-CoA is a key intermediate in the phenylpropanoid pathway, and its synthesis from phenylalanine is described in Figures 8.11 and 8.12. It is a substrate for the enzyme, chalcone synthase (CHS). CHS is found in most plants and provides the precursor for a large number of flavonoids. A cysteine thiolate anion in the enzyme's active site attacks the carbonyl carbon of coumaroyl-CoA, displacing the CoA and binding the coumaroyl moiety (**FIGURES 8.24** and 8.26). The second substrate is malonyl-CoA, which is produced by an acyl-CoA carboxylase (ACC) complex found in the cytoplasm. Unlike the plastid enzyme complex, the ACC complex in the cytoplasm is one large fusion protein. Its mechanism of action is the same as the multisubunit ACC found in chloroplasts; it uses bicarbonate, ATP, and acetyl-CoA to produce the three-carbon acid, malonyl-CoA (see Chapter 6).

Malonyl-CoA coordinates with an asparagine (Asn) residue in the CHS active site,

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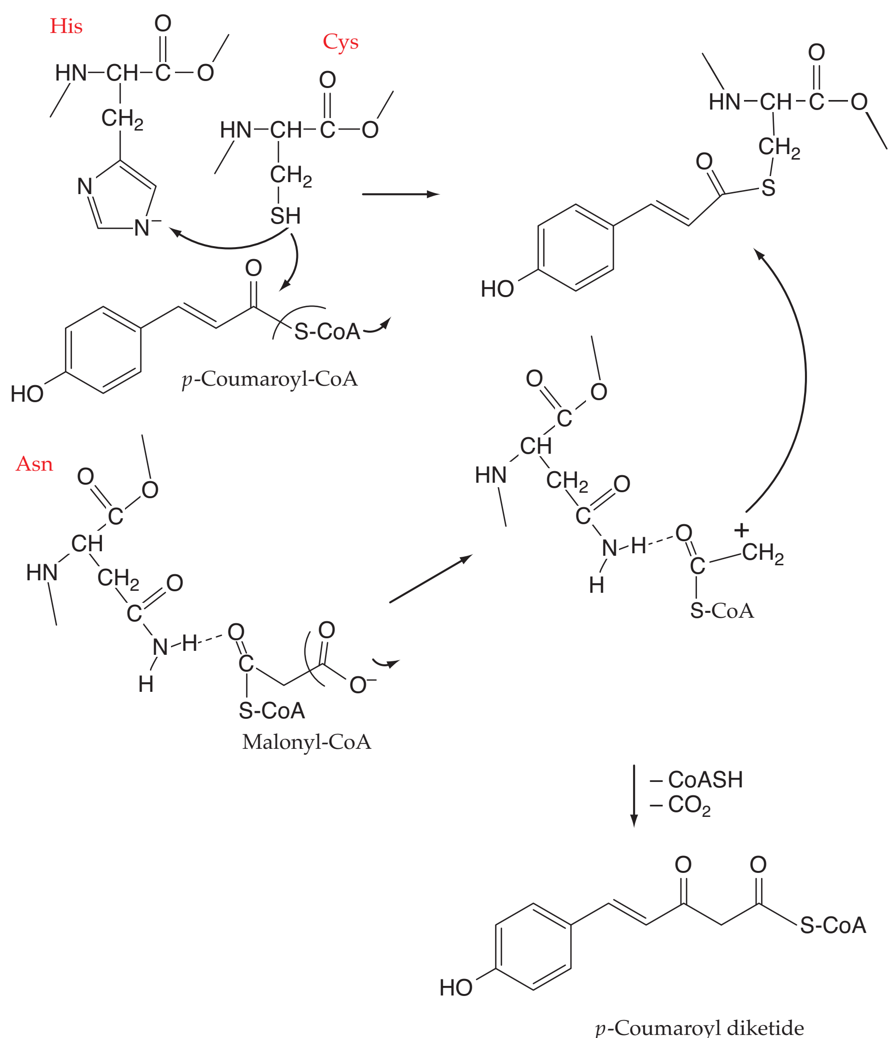

FIGURE 8.24 Mechanism of action of chalcone synthase (CHS). All polyketide synthases have a conserved catalytic triad consisting of histidine (His), cysteine (Cys), and asparagine (Asn) residues in their active sites. The histidine imidazole-N acts as a base and removes a proton from the cysteine sulfhydryl group generating a reactive thiolate anion. This anion attacks the carbonyl carbon of coumaroyl-CoA and displaces the CoA. The second substrate, malonyl-CoA, binds near the asparagine residue. The Asn amide-NH_2 forms an H bond with the carbonyl oxygen of malonyl-CoA and facilitates its decarboxylation. The resulting acetyl cation attacks the carbonyl carbon of the coumaroyl–cysteine complex and produces a diketide product.

which facilitates its decarboxylation. The remaining two-carbon fragment, an acetyl-CoA cation, attacks the carbonyl carbon of the coumaroyl moiety and displaces it from the cysteine active site. The product is coumaroyl diketide (Figure 8.24). This diketide intermediate is reattached to the cysteine thiolate in the active site of CHS and a second equivalent of malonyl-CoA is coordinated to the asparagine residue, decarboxylated, and added to the diketide. The resulting triketide intermediate undergoes another round of binding to the active site cysteine and acetate addition to yield a tetraketide. This final ketide intermediate undergoes cyclization and aromatization while still bound to CHS to produce the product called a chalcone (**FIGURE 8.25**). The overall reaction catalyzed by CHS is as follows:

$$\text{Coumaroyl-CoA} + 3\ \text{Malonyl-CoA} \rightarrow \text{Chalcone} + 4\ \text{CoA-SH} + 3CO_2$$

Chalcone synthases s are found in all vascular plants and some bryophytes. They also occur in fungi and certain classes of algae. Most CHSs have a monomer molecular weight of approximately 40 kDa and function as dimers. The CHSs found in plants are part of a large family of enzymes that can catalyze the synthesis of an aromatic ring from fatty acid precursors, usually malonyl-CoA. The plant synthases are part of a larger superfamily, the polyketide synthases. Polyketides are produced by many organisms, including bacteria such as *Streptomyces,* and

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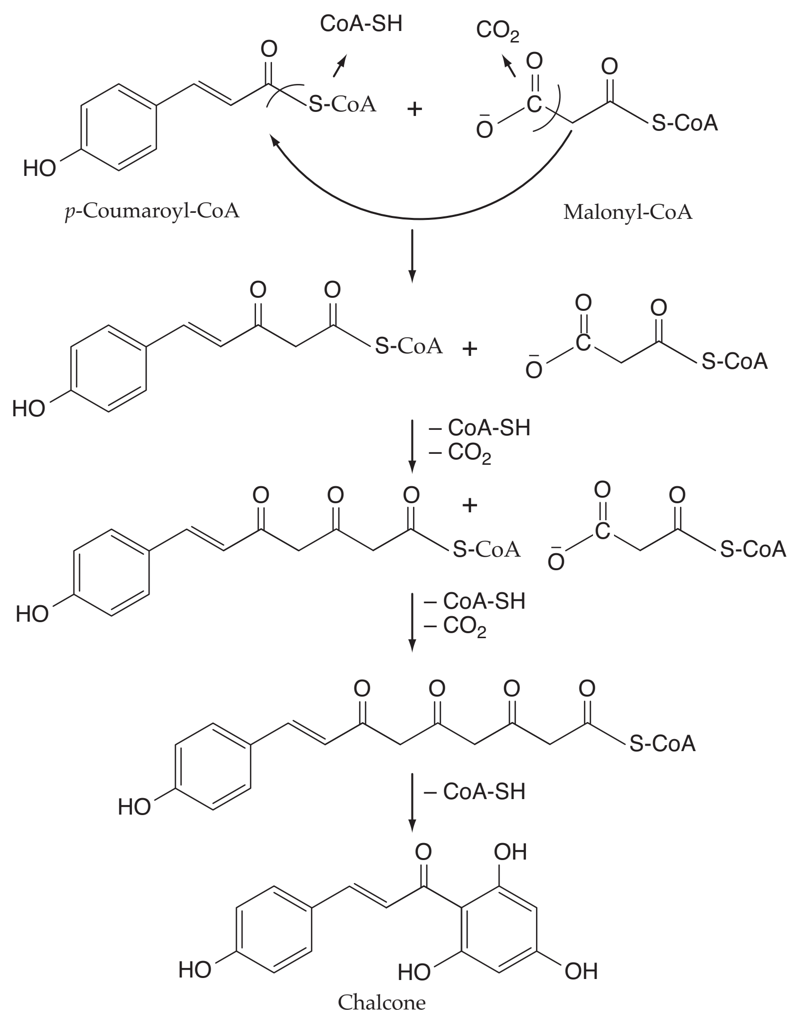
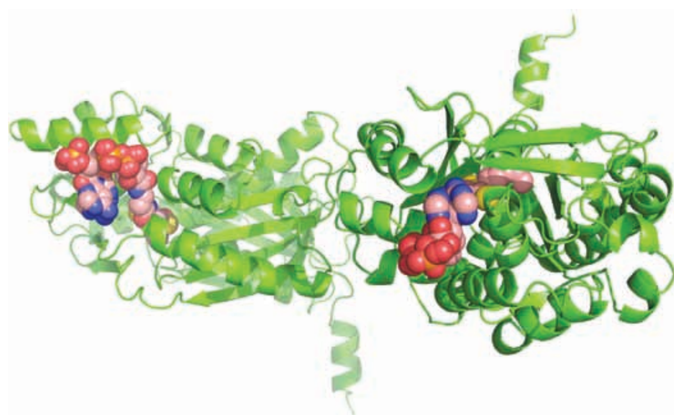


FIGURE 8.25 Chalcone synthesis catalyzed by chalcone synthase (CHS). CHS binds *p*-coumaroyl-CoA first and then adds a two-carbon fragment resulting from the decarboxylation of malonyl-CoA (see Figure 8.24). Loss of CO_2 drives the reaction toward synthesis. The cycle repeats two more times to generate a tetraketide intermediate. The tetraketide undergoes cyclization and generation of double bonds to produce a second aromatic ring in the final product, a tetrahydroxy-chalcone.

form the core structures of certain antibiotics, such as erythromycin. Although they catalyze similar reactions, the polyketide synthases of plants have very little amino acid sequence similarity to their bacterial counterparts. The plant enzymes probably evolved from fatty acid synthases (see Chapter 6).

The x-ray crystal structure of the CHS from alfalfa (*Medicago sativa*) has been solved to 1.5-Å resolution (**FIGURE 8.26A**). The homodimer has two structurally separate active sites. Coumaroyl-CoA binds first in a cleft that excludes other phenolics due to its size and the charged residues in the vicinity of the active site. As shown in **FIGURE 8.26B**, the active site consists of a catalytic triad of histidine, cysteine, and asparagine residues. The histidine acts as a general base and removes a proton from the cysteine sulfhydryl group to produce an active thiolate anion that binds coumarate by displacing the CoA. Malonyl-CoA enters the active site near the asparagine residue, which provides hydrogen bonds to this substrate by way of its amide-NH_2 (γ-amino group). Decarboxylation of malonyl-CoA produces the cation that attacks the coumaroyl moiety, producing a CoA-bound diketide. This cycle is repeated two more times to yield a tetraketide intermediate that then folds and is desaturated to produce an aromatic ring. Naringenin chalcone diffuses out of the active site, which is not large enough to allow further condensation to longer polyketides. The CHS from alfalfa has a high degree of sequence similarity to other polyketide synthases found in plants, including those that produce different products, such as stilbenes and pyrones. Most of the plant synthases seem to have evolved from a common ancestor.


(a)

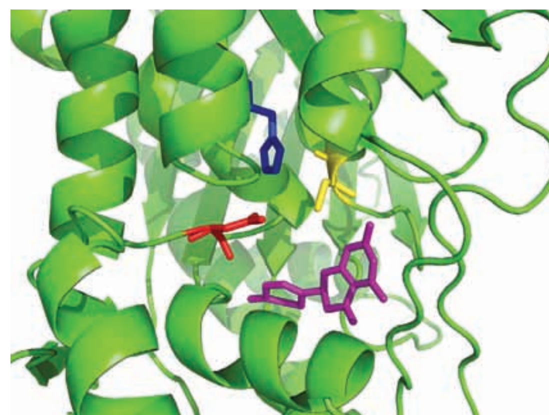

(b)

FIGURE 8.26 X-ray crystal structure of CHS from alfalfa. (a) CHS occurs as a homodimer with two independent active sites. The tunnel into the active site is occupied by a coenzyme A analog shown as spheres. (b) The active site of CHS is shown with a bound narigenin chalcone in purple sticks. The active site histidine-303 (blue) is placed at the top of the binding cavity. It acts as a base to remove a proton from the cysteine-164 shown in yellow. The ketide intermediates are bound to this cysteine residue. Asparagine-336 in red sticks is the binding site for malonyl-CoA. (Structure a from Protein Data Bank 1CHW. J.-L. Ferrer, J. Jez, M. E. Bowman, R. Dixon, and J. P. Noel, *Nat. Struct. Biol.* 6 [1999]: 775–784. Structure b from Protein Data Bank 1CHGK. J.-L. Ferrer, J. Jez, M. E. Bowman, R. Dixon, and J. P. Noel, *Nat. Struct. Biol.* 6 [1999]: 775–784.)

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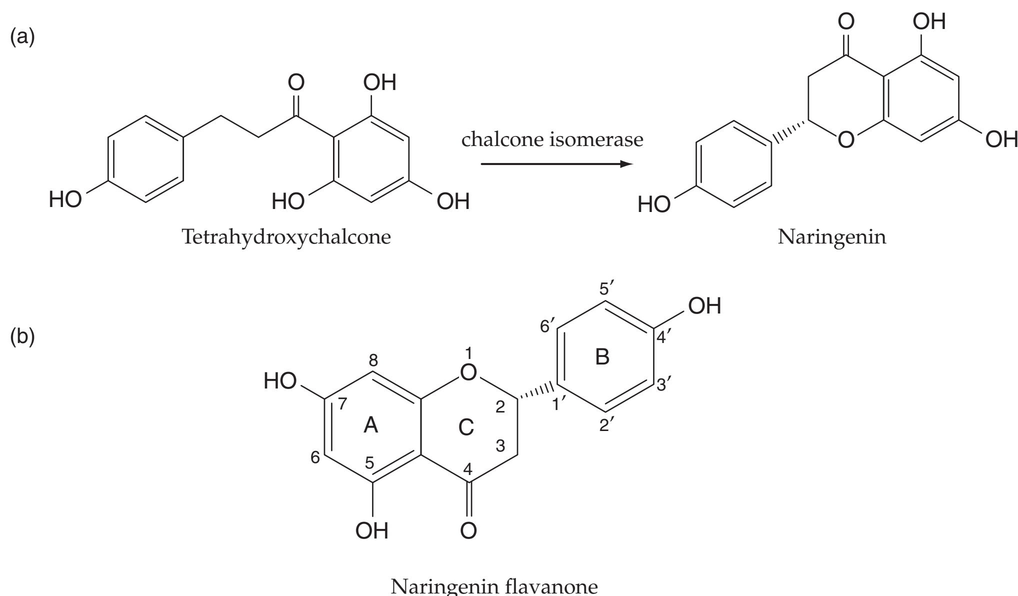

FIGURE 8.27 Synthesis of naringenin flavanone. (a) A specific chalcone isomerase (CHI) catalyzes formation of the pyran ring in the *S*-configuration. (b) The letter designations and numbering system for the general flavonoid molecule are illustrated.

Synthesis of Flavanones and Derivatives

The chalcones are the precursors for most flavonoid compounds. Further ring closure to produce a pyran ring in naringenin occurs spontaneously. Plants, however, have an isomerase that catalyzes this reaction. The enzymatic ring closure is at least 100 times faster than the uncatalyzed reaction and also produces the biologically active *S*-isomer (**FIGURE 8.27**). The uncatalyzed reaction results in a racemic mixture. The product of isomerization is classified as a flavanone and is the key intermediate in the synthesis of other classes of flavonoids. The major differences in these compounds are changes in the oxidation state of the pyran (C) ring (**FIGURE 8.28**).

Many of the modifications shown in Figure 8.28 are catalyzed by oxygenases that add hydroxyl groups or a double bond to the C ring of naringenin flavanone. These enzymes are associated with the outer face of the endoplasmic reticulum. Additional enzymatic reactions can also modify the A and B rings to produce a large array of derivatives that are collectively referred to as flavonoids.

Most flavonoids are glycosylated for storage or transport. The sugar attached to the flavonoid is usually glucose but can also be galactose, arabinose, xylose, or rhamnose. These reactions are catalyzed by glycosyl transferases that use a UDP-sugar substrate and make an ether bond between the sugar and an OH group in the flavonoid. Additional sugars are often added to the first one.

Some flavonoids are acylated, usually with methyl or acetyl groups, but other additions are possible. The acyl groups are added to an OH in one of the rings or to the sugar moiety in glycosylated flavonoids. Acylation can change the solubility of the flavonoids and cause a shift in their absorption spectra.

A sulfotransferase can catalyze the sulfation of the B ring of some flavonoids. The sulfur

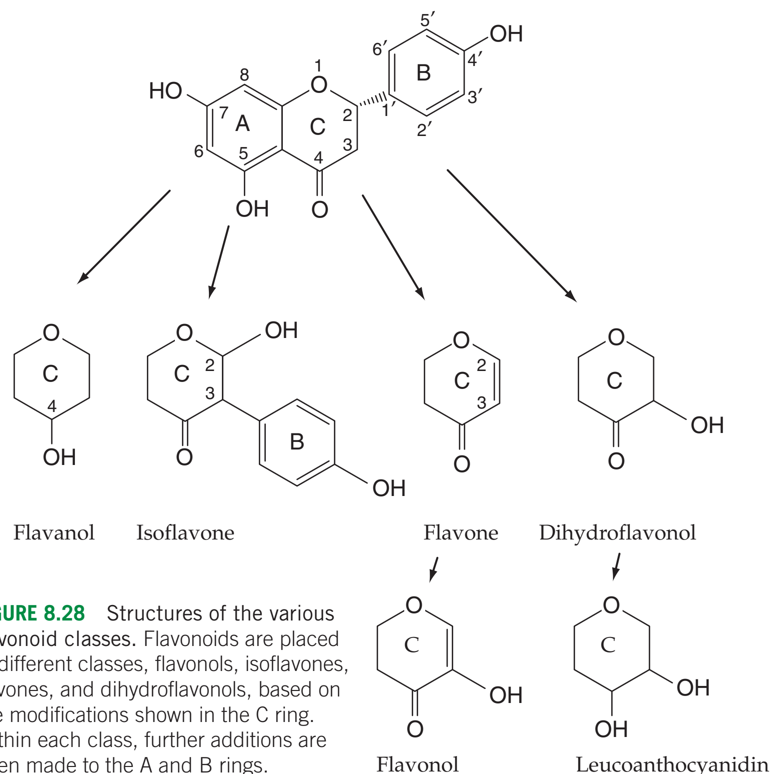

FIGURE 8.28 Structures of the various flavonoid classes. Flavonoids are placed in different classes, flavonols, isoflavones, flavones, and dihydroflavonols, based on the modifications shown in the C ring. Within each class, further additions are often made to the A and B rings.

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donor is phosphoadenosine phosphosulfate (PAPS). Sulfated flavonoids are often signaling molecules used by plants such as legumes to attract the appropriate species of symbiotic bacteria (see Chapter 5).

Flavonoids can be prenylated, usually with dimethylallyldiphosphate but occasionally with longer terpene precursors. This increases the hydrophobicity of the products. Prenylated flavonoids generally function as phytoalexins.

Synthesis and Properties of Flavones

Flavones are made from naringenin flavanone by addition of a double bond to the C-ring between C-2 and C-3 (Figure 8.28). Two different flavone synthases (FNS) can catalyze this desaturation. In many angiosperms, flavone synthase II (FNS II) is a cytochrome P450 monooxygenase. In others, such as some species in the Apiaceae (parsley, carrot), the synthase (flavone synthase I [FNS I]) is an oxoglutarate-dependent dioxygenase (ODD). The ODDs are nonheme iron–containing enzymes that can bind O_2 and transfer one oxygen atom to oxoglutarate (α-ketoglutarate) and the other oxygen atom to a substrate or catalyze the formation of a double bond as in the fatty acid desaturases.

As illustrated in **FIGURE 8.29**, the iron in flavone synthase I is ligated to histidine and aspartic acid residues in the active site. This Fe^{3+} is reduced by ascorbic acid. The ferrous form of the enzyme then binds oxoglutarate and molecular oxygen. The oxoglutarate undergoes oxidative decarboxylation to succinate, which is released from the enzyme. This reaction generates an active ferryl intermediate, and the enzyme can now bind and catalyze the formation of a double bond in the substrate, naringenin flavanone. Additional reactions in flavonoid modification are catalyzed by ODDs, and genomic analyses suggest that this type of dioxygenase is more common than previously thought. The *Arabidopsis* genome has approximately 64 genes for putative ODDs, and some oxygenase-catalyzed reactions may use this type of enzyme rather than cytochrome P450 monooxygenases.

Flavones are colorless compounds with an absorption maximum in the UV region of the spectrum. They are generally stored in cells as 7-O-glycosides and can be found in all tissues of most plants, except for the Brassicaceae, which do not produce flavones. Flavones function to protect plant cells from UV radiation and may also act as allelochemicals (i.e., compounds that inhibit growth of competing photosynthetic organisms). They may also serve to attract and guide pollinators. As seen in **FIGURE 8.30**, colorless flavones fluoresce under UV light, and the patterns can be detected by bees and some other potential pollinators. The flavones serve as a nectar guide but also ensure that pollen is transferred onto the insect.

Synthesis and Properties of Anthocyanidins

Anthocyanidins are the precursors of plant pigments collectively referred to as anthocyanins. Their colorless precursors, leuco-anthocyanidins, are synthesized from dihydroflavonols, which are the products of another ODD that introduces an OH group at C-3 in the C ring of naringenin flavanone. The keto group in the

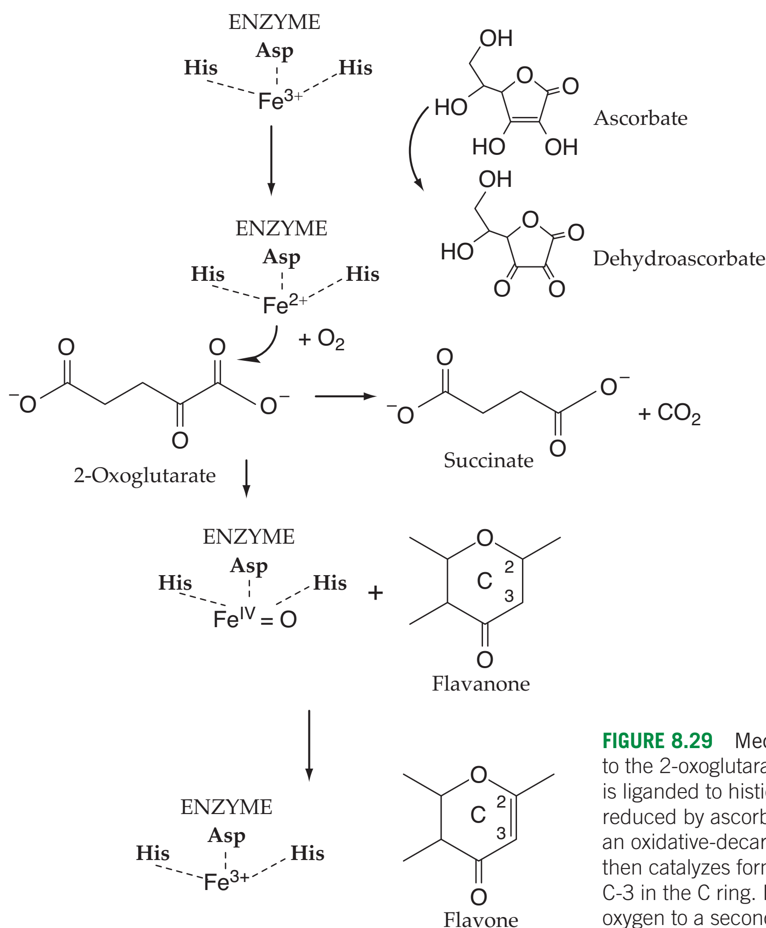

FIGURE 8.29 Mechanism of action of flavone synthase I (FNS I). FNS I belongs to the 2-oxoglutarate–dependent nonheme iron family of dioxygenases (ODD). Iron is liganded to histidine and aspartic acid residues in the protein. The ferric iron is reduced by ascorbate. The Fe^{2+} form of the enzyme binds oxoglutarate and catalyzes an oxidative-decarboxylation to succinate. The resulting $Fe^{IV} = O$ form of the enzyme then catalyzes formation of a double bond in the flavanone substrate between C-2 and C-3 in the C ring. In a more generalized reaction, an ODD transfers the Fe-bound oxygen to a second substrate to generate a hydroxylated product.

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(a)

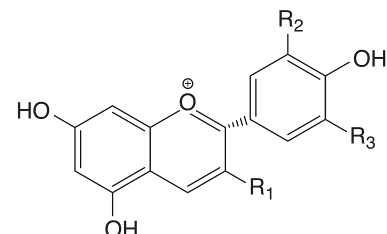
(b)

FIGURE 8.30 Colorless flavones are visible under UV light. Flower (*Coreopsis leavenworthii*, top; *Bidens mitis*, bottom) images are shown photographed under visible (a) and UV (b) light. The UV images are transduced into blue. UV-absorbing flavones are concentrated in the center of the flowers. The patterns are visible to some insects, such as honeybees, and may increase attraction to the flowers and help guide the pollinator after it lands.

C ring is then reduced, and a second ODD, anthocyanidin synthase, introduces two double bonds into the ring to produce the anthocyanidin flavylium cation (**FIGURE 8.31**). The final steps in anthocyanin synthesis include glycosylation of the OH group in the C ring and further modifications of the A and B rings.

The unmodified anthocyanidin flavylium cation can be generated *in vitro* and exhibits a reddish hue at neutral pH. The color is influenced both by the type of solvent and pH. In flower petals the color of the anthocyanins can vary depending on the chemical modifications, including further glycosylation, acylation, and prenylation. Anthocyanins are often stored in special vacuoles modified for pigment storage. These vacuoles are acidic and generally give rise to a blue color. Changes in the vacuolar pH, such as raising the pH to 7 or higher, can shift the color toward the red end of the spectrum. Changes in cell shape influence stacking of the aromatic rings of the anthocyanins and lead to variations in the perceived colors. The presence of cations, mainly Mg^{2+} and Al^{3+}, can also influence the final color of flower pigments. A particularly dramatic example are the colors of

FIGURE 8.31 Examples of anthocyanidins. The product of anthocyanidin synthase is the flavylium cation shown at the top that is further modified to produce different colored products. The hydroxyl (OH) group at R_1 in most anthocyanidins is glycosylated to stabilize the ring structure. Hydroxyl groups in the A and B rings are also often glycosylated. The colors shown above can be generated *in vitro* from purified compounds. The actual colors of anthocyanidins *in vivo* depend on a number of physiological parameters such as pH of the compartment in which the pigments are stored and presence of metal ions and copigments.



Pelargonidin: R_1 = OH, R_2 = H, R_3 = H
Luteolinidin: R_1 = H, R_2 = OH, R_3 = H
Delphinidin: R_1 = OH, R_2 = OH, R_3 = OH
Cyanidin: R_1 = OH, R_2 = OH, R_3 = H

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hydrangea flowers, which are generally pink when grown in alkaline soils but can change to blue after application of an acidic mixture of soluble aluminum salts to the soil (see photo on page 119). The presence of other flavonoids, such as the colorless flavones, act as copigments and can also modulate color. Anthocyanins in flowers can obviously have a variety of colors and are a major factor in the attraction of pollinators. Genetic engineering techniques have been used to try to alter flower colors for commercial purposes. Given the number of factors that can influence the final petal colors, this is not readily accomplished by modifications of a few genes.

In contrast to flower petals, anthocyanins in leaves are usually a red color and are responsible for the bright red of autumn leaves, particularly in some maple species. Anthocyanins in photosynthetic tissues are stored in vacuoles and protect cells against the damaging effects of intense sunlight. Their synthesis can be increased during periods of high light intensity and low temperatures, such as early spring in the northern hemisphere. Under these conditions, the light absorption by anthocyanins reduces the rate of the light absorption and transfer in photosystem II. In cool conditions, CO_2 fixation is limiting and cannot recycle the cofactors required in the light reaction. The attenuation of oxygen evolution in photosystem II by anthocyanins helps prevent the formation of oxygen free radicals. The anthocyanins themselves are also very effective antioxidants. They can readily dissipate reactive oxygen species such as the hydroperoxy radical and other radical species produced in peroxisomes (see Appendix 5).

Anthocyanidins can be polymerized into oligomers called proanthocyanidins or condensed tannins. The precursors accumulate in vacuoles and are exported to the cell wall. Subsequent oxidation by laccases produces linear polymers with a deep purple to brown color characteristic of some seeds, fruits, and tree bark. These compounds inhibit proteolytic enzymes in herbivores and also slow the growth of bacterial and fungal pathogens.

Synthesis and Properties of Isoflavonoids

Isoflavonoids are synthesized from naringenin flavanone by a cytochrome P450 monooxygenase. Isoflavone synthase (IFS) catalyzes the cleavage of the C–H bond at C-3 in the C ring and generates a radical intermediate. The B ring then migrates to C-3 and the enzyme adds an OH group to C-2 (**FIGURE 8.32**). A dehydratase can remove H_2O from this initial product to yield a specific isoflavone such as genistein. Isoflavone synthase can also act on deoxyflavonones that lack the hydroxyl group on C-5 of ring A to produce an additional class of isoflavonoids. As in the other flavonoids, modifying groups such as sugars can then be added to the basic isoflavonoid ring system.

Isoflavonoids are not widespread among plants but are commonly found in legumes. Isoflavonoid synthesis is inducible, and the products act as phytoalexins in leaf tissues to protect against pathogens. Isoflavonoids are excreted from legume roots and serve as attractants for nitrogen-fixing symbiotic bacteria (see Chapter 5).

The isoflavone ring system bears a resemblance to the mammalian sex hormones, the estradiols (**FIGURE 8.33**). Isoflavonoids are able to bind weakly to mammalian estrogen

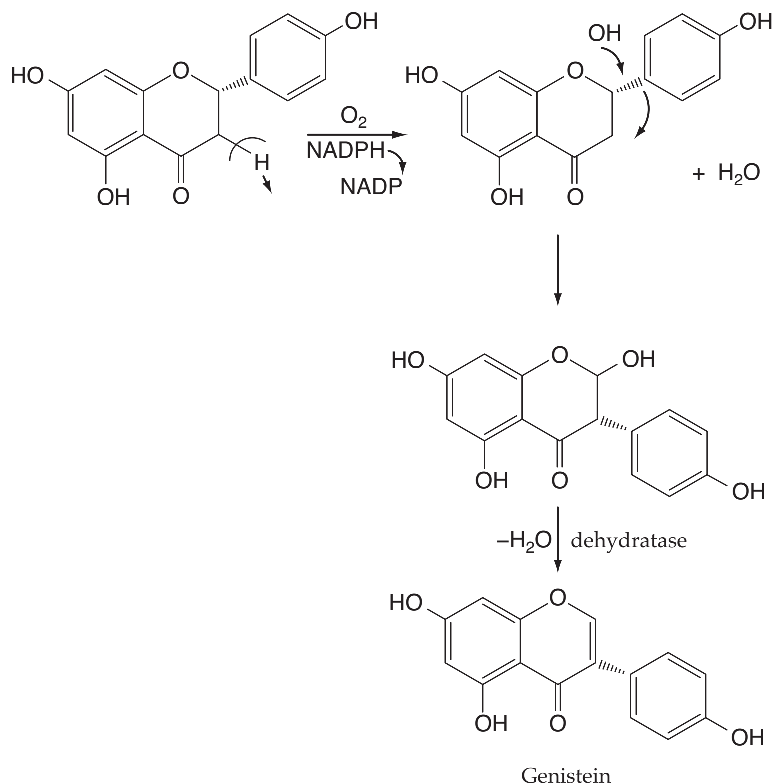

FIGURE 8.32 Synthesis of genistein isoflavone. The enzyme, isoflavone synthase, is a specific cytochrome P450 monooxygenase. It binds the S-isomer of naringenin flavanone and catalyzes removal of an H atom at C-3 in the C ring, producing a radical intermediate. Migration of the aromatic B ring to C-3 and hydroxylation of C-2 yield an isoflavone intermediate. In soybean (*Glycine max*), a dehydratase removes H_2O from the C ring, producing the isoflavone, genistein.

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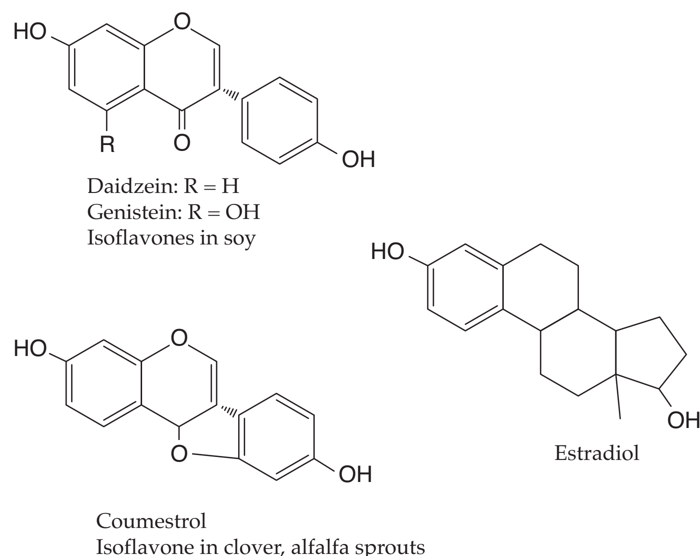

FIGURE 8.33 Some isoflavonoids resemble estradiol. The ring structure of isoflavones in many legumes resembles the mammalian sex hormone, estradiol, and can bind to estrogen receptors. The critical property for receptor binding is the distance between the hydroxyl groups.

receptors and can function as phytoestrogens. The phytoestrogen in clover, coumestrol, is known to have negative effects on the reproductive cycles of ruminants such as sheep. The phytoestrogens in soy-rich foods, such as tofu, may have beneficial effects in humans. Clinical trials have shown that a soy-rich diet can reduce blood cholesterol levels and the risk of heart attacks in postmenopausal women. The U.S. Food and Drug Administration recommends an intake of 25 g per day of soy protein to diminish the effects of menopause and reduce cholesterol levels. The effects are variable and may depend on the activity of gut flora that can modify the phytoestrogens and promote uptake from the intestines.

Examples of Other Plant Polyketide Synthases

CHS is ubiquitous in plants and provides the naringenin precursor for a wide variety of flavonoids. Other types of polyketide synthases have been described that are often species specific, leading to the production of unique natural products. Only a few of these are described here to illustrate how small changes in enzyme structure can lead to a variety of different products. One example is the synthesis of curcumin in *Curcuma longa*, a plant in the Zingiberaceae (ginger) family. In *C. longa*, a unique diketide synthase adds malonyl-CoA to the CoA ester of ferulic acid (**FIGURE 8.34**). A second feruloyl-CoA is added to the diketide product to yield curcumin, the main ingredient in the spice, turmeric. Curcumin is produced mainly in the roots of the plant as a phytoalexin. Humans use the roots to provide a pungent taste and yellow color to foods. Curcumin also inhibits the secretion of mucus in mammalian lungs and may be useful in treatment of the symptoms of asthma and cystic fibrosis.

Pyrone Synthases The pyrone synthases are another type of polyketide synthase with restricted distribution among plants. The basic pyrone synthase uses acetyl-CoA as its initial substrate and adds malonyl-CoA in a condensation reminiscent of fatty acid synthesis (**FIGURE 8.35**). Pyrone synthases catalyze the production of a triketide intermediate and its subsequent cyclization to 6-methyl-4-hydroxy-pyrone.

Like the CHSs, the pyrone synthases are dimers with two active sites. The chalcone and basic pyrone synthases exhibit 30% to 50% amino acid similarity and the same catalytic triad of histidine, cysteine, and asparagine. A comparison of the x-ray crystal structures of CHS from *Medicago sativa* and the pyrone synthase from *Gerbera hybrida* is shown in **FIGURE 8.36**. The structural data clearly illustrate that production of different products is a result of minor residue mutations that affect the size and hydrophobicity of the ketide binding cavity.

Stilbene Synthase An especially perplexing example of polyketide synthesis is the production of stilbenes.

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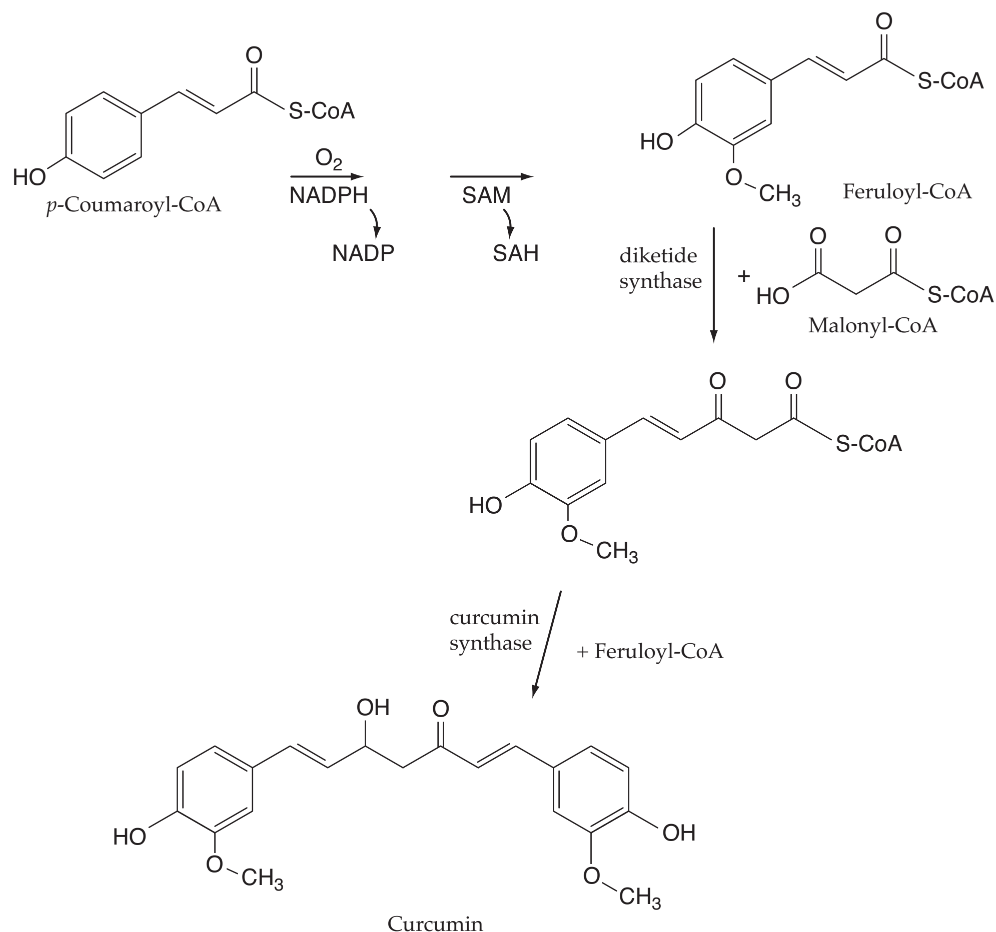

FIGURE 8.34 Synthesis of curcumin. A unique diketide synthase binds the CoA ester of ferulic acid (see Figure 8.14) and catalyzes the addition of malonyl-CoA. The feruloyl-CoA diketide intermediate is further modified by a specific polyketide synthase that adds a second feruloyl moiety to the side chain to yield curcumin. This particular polyketide synthase is unique to some plants of the Zingiberaceae (ginger) family.

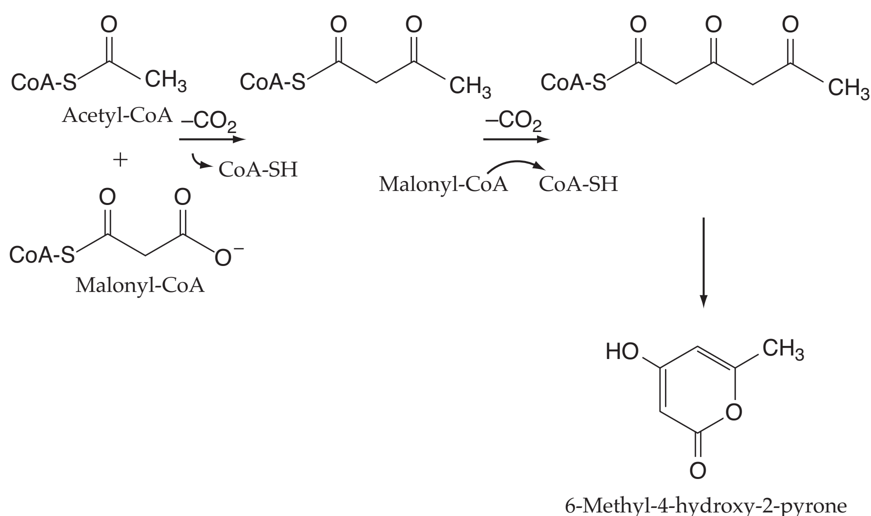

FIGURE 8.35 Activity of pyrone synthase. The enzyme catalyzes the sequential condensation of two molecules of malonyl-CoA with an acetyl-CoA starter unit instead of the *p*-coumaroyl-CoA starter in CHS. A triketide intermediate is produced in this reaction and then cyclized into an unsaturated δ-lactone called a pyrone.

Stilbene synthases utilize coumaroyl-CoA as their initial substrate and add three units of malonyl-CoA to produce the same tetraketide intermediate that is found in chalcone synthesis. The stilbene synthases, however, catalyze a subsequent decarboxylation of the intermediate and a C-2 to C-7 cyclization instead of the C-1 to C-6 cyclization in chalcone synthases (**FIGURE 8.37**). Both types of synthases exhibit over 50% amino acid similarity and have identical residues in the active site and surrounding substrate entrance cavity. Yet they consistently catalyze the formation of different products from the same tetraketide intermediate. Subtle differences in the active site region apparently favor the formation of a larger molecule by CHSs and decarboxylation of the tetraketide and synthesis of a smaller product by stilbene synthases.

As in the case of the flavonoids, the stilbenes can be further modified, mainly by glycosylation.

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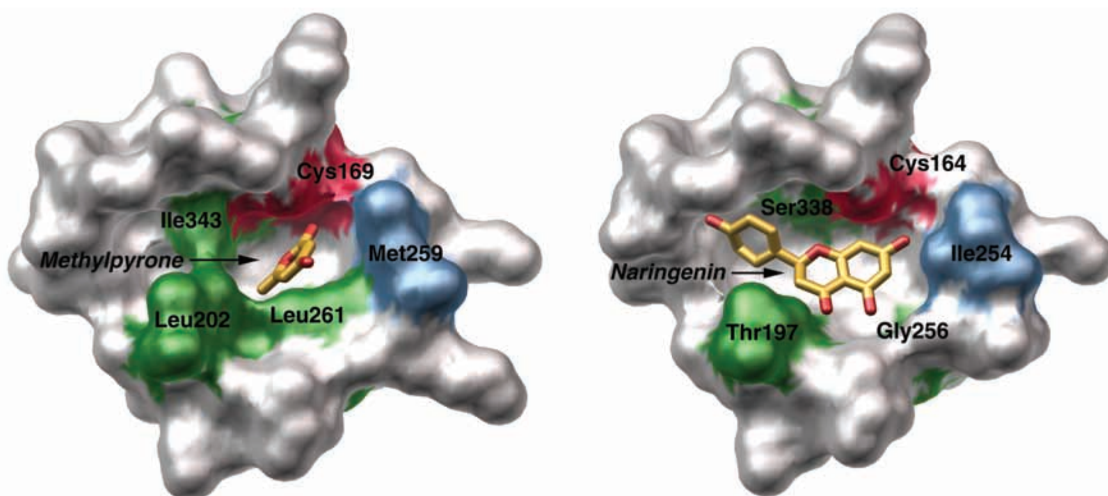

FIGURE 8.36 Comparison of the active site structures of 2-pyrone synthase (2-PS) (left) and chalcone synthase (CHS) (right). The major difference between these homologous, dimeric enzymes is the size of the binding cavity, which is considerably smaller in the case of 2-PS. In addition, 2-PS has an active site entrance that is constricted compared with CHS and lined with several hydrophobic residues. Relatively small molecules have access to the active site cavity and result in the condensation and exit of relatively hydrophobic products. This is in contrast to the more open and hydrophilic cavity of CHS (see also Figure 8.26). (*Reproduced from*: J. M. Jez, M. B. Austin, J. Ferrer, M. E. Bowman, J. Schröder, and J. P. Noel, *Chemistry & Biology* 7 [2000]: 919–930, Figure 6. Courtesy of Joseph P. Noel, Howard Howard Hughes Medical Institute.)

They are widespread but not universally distributed in flowering plants. They have antibacterial and antifungal properties and act as phytoalexins. Resveratrol, the stilbene shown in Figure 8.37, is abundant in grapes. Based on experiments *in vitro* and experiments in animals, it acts as an antioxidant and can inhibit blood platelet aggregation (clotting) in mammals. Resveratrol is the compound in red wine that is presumably responsible for the "French paradox," that is, how to consume a diet rich in saturated fats and cholesterol and still maintain a low risk for atherosclerosis and heart attack. Experiments in mice have recently shown that consumption of resveratrol protects mice from the adverse effects of a high-calorie diet even though the animals were relatively obese compared with controls. Larger quantities of resveratrol than are present in a normal diet, however, must be consumed to obtain these health benefits. Resveratrol is available in pure form and clinical trials are currently being done in humans suffering from type 2 diabetes.

Synthesis and Activity of Coumarins

The coumarins, like other products of the phenylpropanoid pathway, start with the synthesis of the key intermediate, coumaroyl-CoA. Coumarin synthesis requires a specific hydroxylase (cytochrome P450 monooxygenase) that adds an OH to the aromatic ring at the *ortho* position. The next step is a nonenzymatic isomerization of

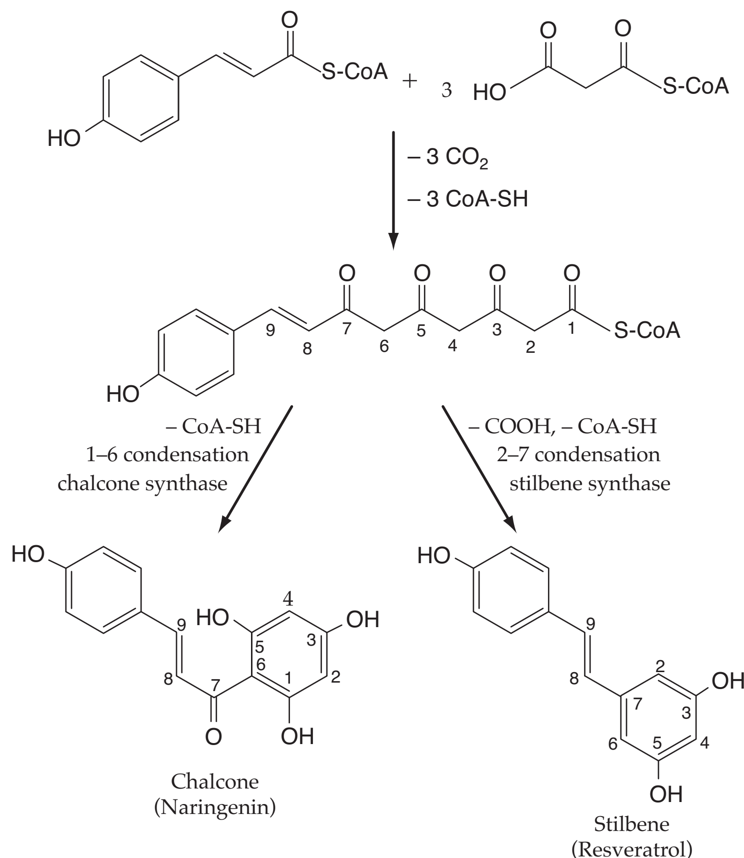

FIGURE 8.37 Comparison of the activity of chalcone and stilbene synthases. Both enzymes catalyze the condensation of three molecules of malonyl-CoA with a *p*-coumaroyl-CoA staring unit to generate the same tetraketide intermediate. In chalcone synthase CHS, the intermediate undergoes a C-1 to C-6 cyclization and aromatization to produce naringenin chalcone. In stilbene synthase, the tetraketide intermediate undergoes a decarboxylation before cyclization of C-2 to C-7 to produce the second aromatic ring to yield resveratrol.

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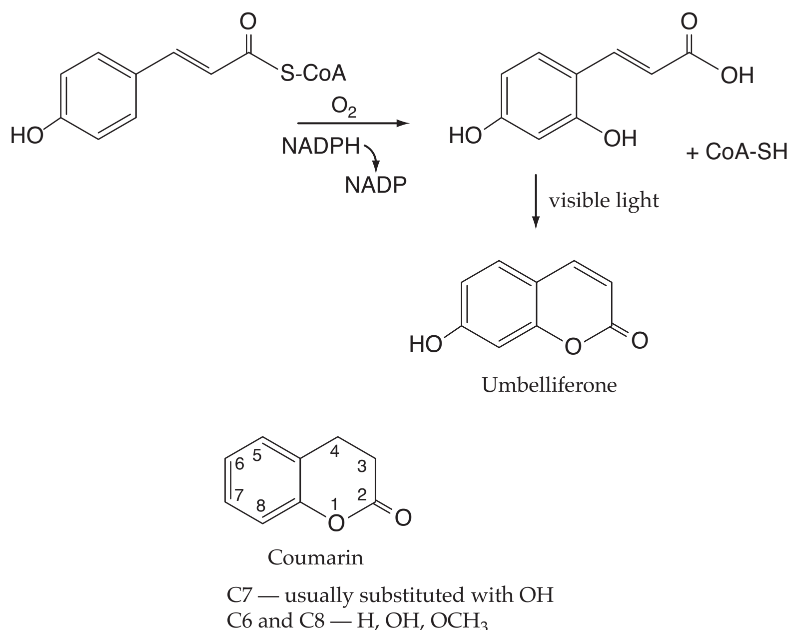

FIGURE 8.38 Synthesis of umbelliferone. Coumarins are synthesized from *p*-coumaroyl-CoA, the key phenylpropanoid pathway intermediate. A specific cytochrome P450 monooxygenase hydroxylates the ring at the *ortho* position with respect to the side chain and removes the CoA. A light-catalyzed isomerization of the double bond in the side chain is followed by spontaneous formation of the δ-lactone. Coumarins can be further modified by hydroxylation and methylation. The inset shows the numbering system and substitution patterns of coumarins.

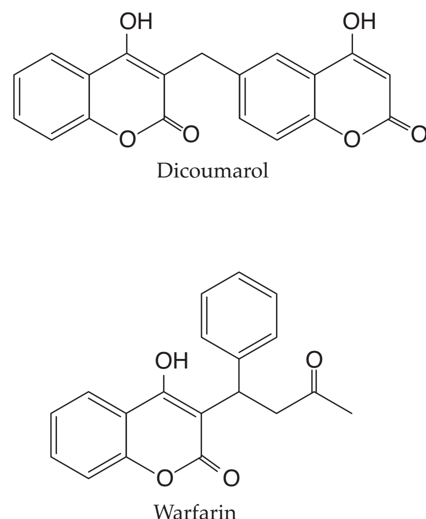

FIGURE 8.39 Structures of two coumarin derivatives. Dicoumarol is produced in damaged clover plants by dimerization of coumarol. It is a powerful anticoagulant in mammals. Warfarin is a synthetic coumarin with structural similarity to dicoumarol and is used therapeutically to prevent internal clot formation.

the double bond in the side chain catalyzed by visible light, followed by spontaneous formation of a δ-lactone. **FIGURE 8.38** illustrates the synthesis of umbelliferone and the general structural designations for coumarins.

Coumarins have a restricted distribution among angiosperms and are found in some members of the Fabaceae, Asteraceae, and Apiaceae. The sweet smell of new-mown hay is due to the release of coumarins from damaged clover (*Melilotus officinalis*). Coumarins can be dimerized to dicoumarol, which is a highly effective anticoagulant (**FIGURE 8.39**). Consumption of large amounts of sweet clover can cause internal bleeding in ruminants. Synthetic anticoagulants, such as warfarin, are based on the dicoumarol structure. Once widely used as a rat poison, warfarin is now prescribed as an anticlotting agent for the treatment of humans with atherosclerosis.

Coumarins are often modified by the addition of other chemical groups. The C-7 position is usually hydroxylated and glycosylated. A specific prenyl transferase can use dimethyl allyl diphosphate to prenylate C-6. The prenylated coumarin is then further modified by two monooxygenases to yield a linear furano derivative called a psoralen (**FIGURE 8.40**).

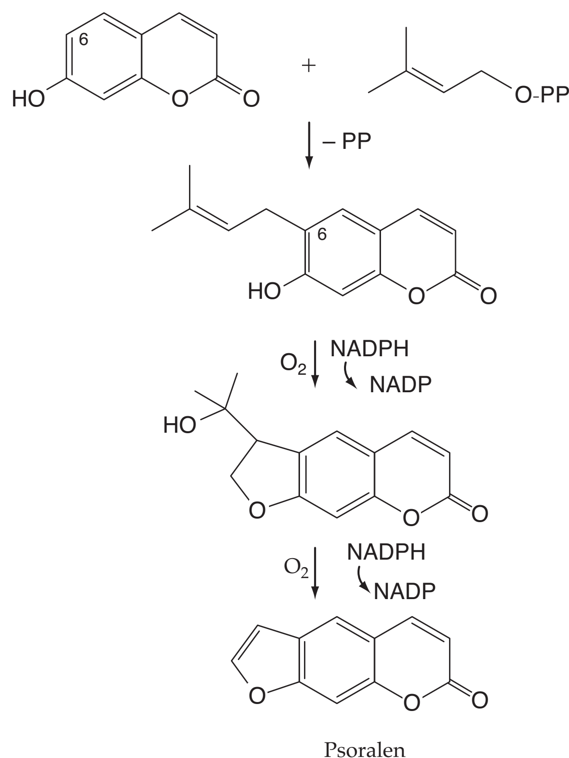

FIGURE 8.40 Synthesis of the furanocoumarin, psoralen. Umbelliferone (7-hydroxycoumarin) is prenylated at C-6 with dimethylallyldiphosphate, which can then lead to formation of a five-membered furan ring. The prenyl side chain is hydroxylated by a cytochrome P450 monooxygenase. A second P450 monooxygenase catalyzes removal of the side chain and formation of a double bond in the furan ring to form psoralen. The ring may be modified by glycosylation. The linear furanocoumarins are phytophotosensitizers that cause severe dermatitis in humans.

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The furanocoumarins are produced in some crop plants such as celery in response to fungal pathogens. These coumarin derivatives act as phytophotosensitizers in humans. Workers who harvest the plants by hand can come in contact with the furanocoumarin; subsequent exposure of the skin to sunlight causes dermatitis followed by severe blistering of the skin. In the United States, cow parsnip or hogweed (*Heracleum sphondylium*) is an invasive plant that produces furanocoumarins. The plant is often found growing in open fields and along highways. Its dermatitis-causing properties present a major barrier to removal and eradication of this invasive species.

CHAPTER SUMMARY

A general outline describing the synthesis of aromatic compounds in plants is illustrated in **FIGURE 8.41**. Synthesis begins in plastids with two simple intermediates of primary carbon metabolism, erythrose-4-phosphate and phosphoenolpyruvate (PEP). These feed into the shikimic acid pathway to produce chorismic acid and the essential aromatic amino acids, tyrosine, phenylalanine, and tryptophan. Intermediates in the shikimic acid pathway can provide the building blocks for additional aromatics such as the gallotannins and the plant hormone, salicylic acid (Figure 8.1). Phenylalanine is the central product of the shikimic acid pathway; it is the substrate for phenylalanine ammonia lyase (PAL), a cytoplasmic enzyme and the gateway to phenylpropanoid synthesis. The precursor for most phenylpropanoids is coumarate or an esterified derivative, most often coumaroyl-CoA. The synthesis of primary products such as lignin draws from the pool of coumaroyl-CoA. Natural products such as flavonoids also draw from the same precursor pool and can be considered primary metabolites because of their essential functions in plant–environmental interactions. Many polyketide synthases use coumaroyl-CoA as a starting unit and produce unique natural products such as curcumin (Figure 8.34).

Additional aromatic compounds are synthesized by branches from the phenylpropanoid pathway, and only a few of these have been covered in this chapter such as the coumarins. Aromatic metabolism in plants comprises a rich assortment of chemical reactions and products. The structures and syntheses of many new compounds are still not fully resolved and others are waiting to be discovered. The aromatic amino acids are also precursors in other unique synthetic pathways, such as the glucosinolates covered in Chapter 5. The alkaloids are a rich assortment of additional natural products that are also derived from amino acids, particularly the aromatic amino acids. A small number of these are described in Chapter 9.

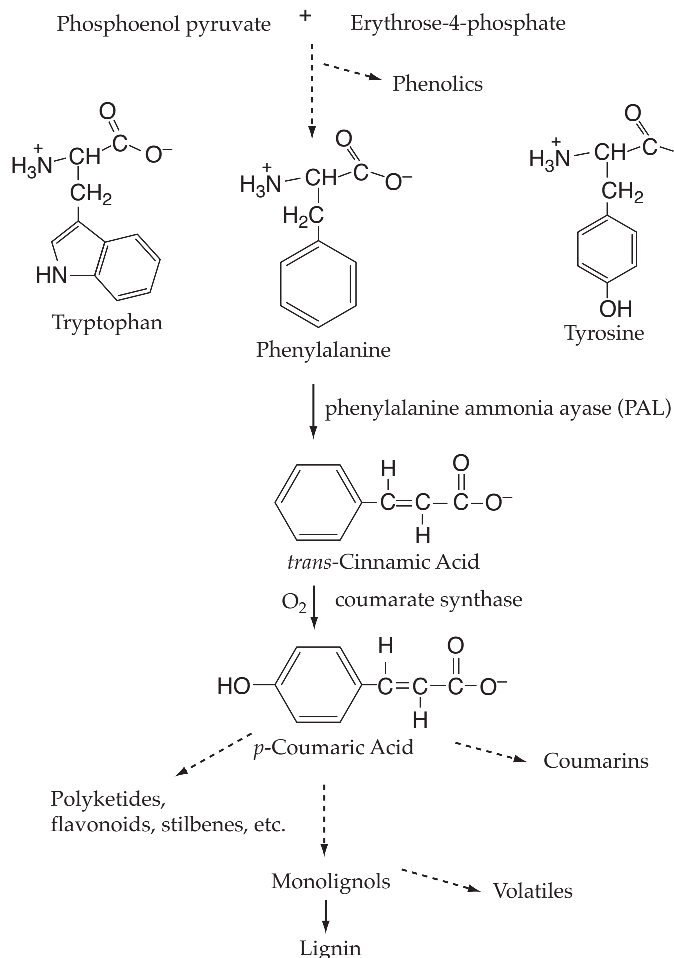

FIGURE 8.41 Summary of general aromatic metabolism in plants. The synthesis of aromatic compounds starts in plastids with the shikimic acid pathway and results in the production of the three aromatic amino acids, tyrosine, phenylalanine, and tryptophan. Phenylalanine occupies a key position as the precursor of the phenylpropanoid pathway. The cytoplasmic enzyme, PAL, removes the amino group from phenylalanine and yields *trans*-cinnamic acid, which is a substrate for a cytochrome P450 monooxygenase. The product is *p*-coumaric acid, which can be further modified, usually by esterification to coenzyme A. The resulting pool of coumaroyl-CoA in the cytoplasm is a source for the synthesis of monolignols and lignin, which are primary products, and a large number of natural products, including the vital flavonoids and their derivatives and defensive compounds such as coumarins.

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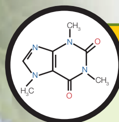

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