1.28
Isoflavonoids: Biochemistry, Molecular Biology, and Biological Functions

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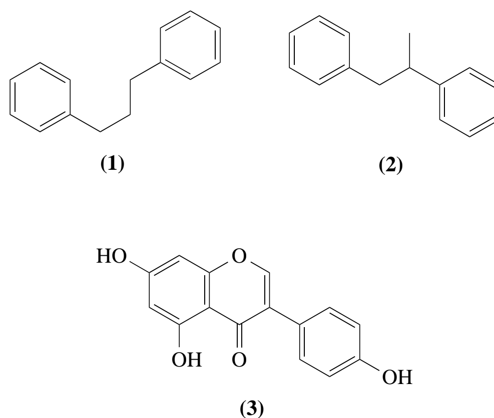
1.28.1 INTRODUCTION: CHEMICAL CLASSES AND BIOLOGICAL OCCURRENCE OF ISOFLAVONOIDS

The flavonoids represent one of the major classes of phenylpropanoid-derived compounds. The 15-carbon (C_6—C_3—C_6) backbone of the flavonoids can be arranged as a 1,3-diphenylpropane skeleton (flavonoid nucleus) (**1**) or as a 1,2-diphenylpropane skeleton (isoflavonoid nucleus) (**2**). More than 4000 different 1,3-diphenylpropane flavonoid derivatives have been characterized from terrestrial plants, in which such flavonoids are almost ubiquitous. In contrast, the isoflavonoids are restricted primarily to leguminous plants, although they occur rarely in other families such as the Apocynaceae, Meliaceae, Pinaceae, Polygalaceae, Compositae, and Myristicaceae.[1] The occurrence of an ester of the isoflavone genistein (**3**) has been reported in *Cotoneaster* (Rosaceae).[2]

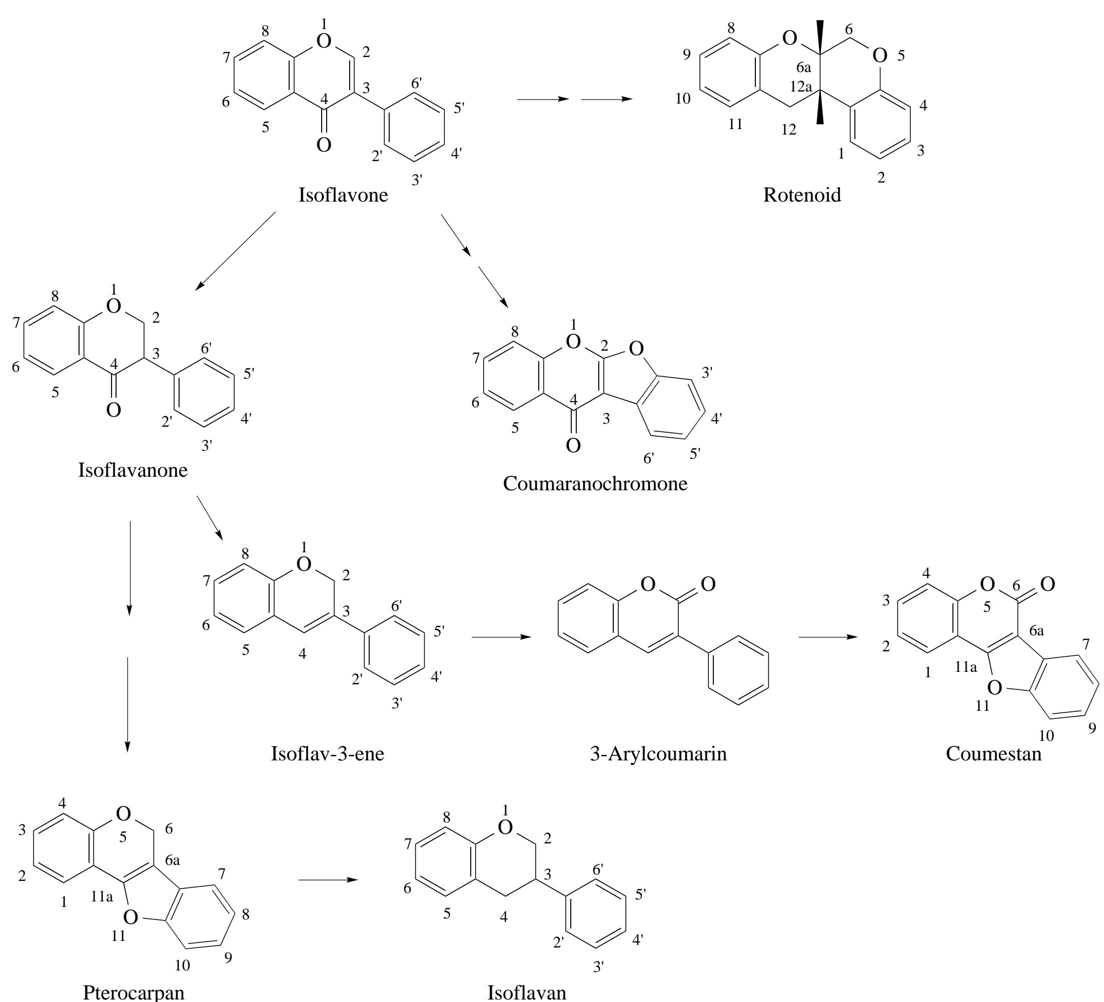


In a review, Tahara and Ibrahim[1] described the classification of 870 naturally occurring isoflavonoid aglycones into nine major classes based on their skeletal modifications. These classes, and their proposed biosynthetic interrelationships, are outlined in Scheme 1, which also indicates the numbering systems used for the different classes of isoflavonoids. Of these classes, the isoflavones and pterocarpans are the most abundant, with 334 and 152 different structures, respectively, having been described as of September 1994.

The limited taxonomic distribution of the isoflavonoids is linked to the occurrence of the enzyme isoflavone synthase, which catalyzes the aryl migration reaction (a two-step process involving hydroxylation/aryl migration followed by dehydration) that leads to the formation of an isoflavone from a flavanone (see below). Many of the subsequent ring modifications that occur in isoflavonoids (e.g., *O*-methylation, isoprenylation, methylenedioxy bridge formation) are also common in the flavonoids *per se*, and are catalyzed by highly regiospecific enzymes with tight substrate (e.g., isoflavonoid class) specificity.

Within a particular species, several different isoflavonoids usually occur. These may be of different classes, and with a variety of substitution patterns. Furthermore, some may be formed constitutively in various plant organs and tissues as part of the plant's developmental program, whereas others are synthesized *de novo* in response to biotic and abiotic stress. Compounds that are synthesized constitutively in a limited range of tissues may accumulate in most tissues of the plant if that tissue is microbially infected. Members of several of the isoflavonoid classes are commonly found in the bark or heartwood of tropical leguminous trees, where they may act as protective compounds. The stress-inducible isoflavonoids with antimicrobial activity (phytoalexins) have been the most studied of the isoflavonoids with respect to biosynthesis, and discussion of these compounds will form the basis of much of the present chapter. Lima bean (*Phaseolus lunatus*) exhibits one of the most varied inducible isoflavonoid responses, with a report of 25 different compounds formed in response to abiotic elicitation.[3]

Compounds (**4**)–(**16**) illustrate the diversity of isoflavonoids that have been reported from the forage legume alfalfa (*Medicago sativa* L.). Glycosides and malonyl glycosides of the isoflavones daidzein (**4**) and formononetin (**5**), and of the pterocarpan medicarpin (**6**), occur constitutively, primarily in root tissue, whereas medicarpin aglycone and the isoflavans sativan (**7**) and vestitol (**8**) are the predominant phytoalexins. The remainder of the compounds shown are minor isoflavonoids that have been identified from large-scale purifications.[4–7] Note that the major ring substituent in alfalfa isoflavonoids is the methoxyl group. In contrast, the major phytoalexins from bean (phase-

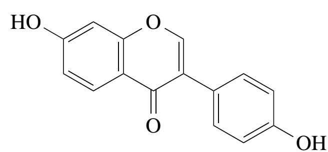


Scheme 1

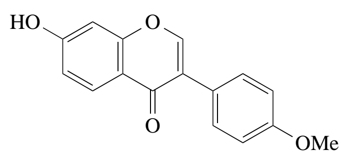
ollin (**17**)) and soybean (the glyceollins I, II and III (**18–20**, respectively)) bear prenyl substituents, whereas (+)-pisatin (**21**) from peas, the first phytoalexin to be structurally characterized, bears methylenedioxy, methoxy, and 6a-hydroxyl substituents. 6a-Hydroxylation of pterocarpans is a common substitution, also occurring in the glyceollins and in the phytoalexins of red clover,[8] among others. Hydroxylation at the 6a-position is also employed by fungi as an early step in the detoxification of pterocarpan phytoalexins.[9]

Pea (*Pisum sativum*) tissues exposed to the biotic elicitor copper chloride have been reported to accumulate, in addition to (**21**), several isoflavones including afrormosin (**22**), (+)-2-hydroxypisatin (**23**), (−)-pisatin (**24**), and the pterocarpene anhydropisatin (**25**).[10–12] The minor compound (**23**) has less antifungal activity than (**21**), but, unlike (**21**), suppresses alfalfa seed germination,[12] suggesting that it might have allelochemical activity.

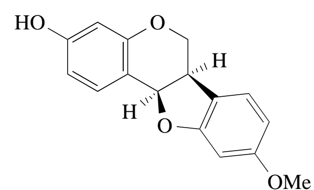
In addition to the commonly occurring isoflavonoids with the typical substituents shown in Scheme 1 and (**4**)–(**16**), more complex isoflavonoids have also been described. Some of these are shown in (**26**)–(**31**). They include rare heteroatom-containing 4′-aminoisoflavonoids from the root bark of *Piscidia erythrina* (reviewed in reference 1) (**26, 27**), the lupinols (coumaranochroman-4-ones) from white lupin (reviewed in reference 1) (**28**), a range of isoflavonoid oligomers (isoflavan–isoflavan such as vestitol-(4→5′)-vestitol (**29**) from heartwood of *Dalbergia odorifera*, isoflavan–isoflavone, isoflavan–flavanone, isoflavan–flavone, isoflavone–flavone, isoflavan–flavene, isoflavone–cinnamyl alcohol, isoflavone–stilbene, and isoflavan–chalcone) (reviewed



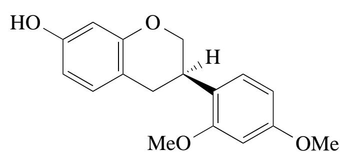
(4) Daidzein



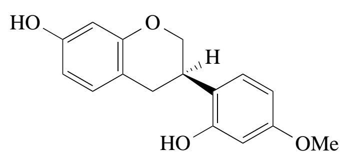
(5) Formononetin



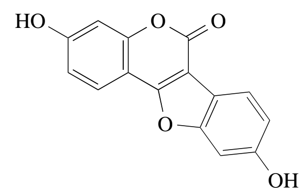
(6) (–)-Medicarpin



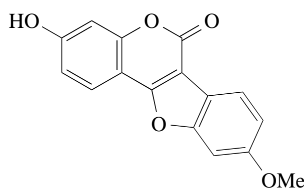
(7) (–)-Sativan



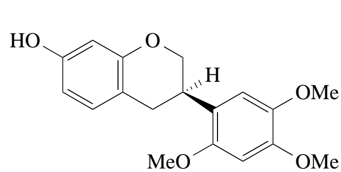
(8) (–)-Vestitol



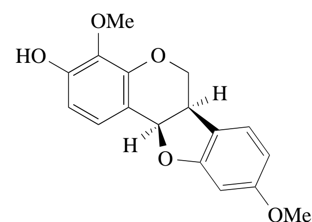
(9) Coumestrol



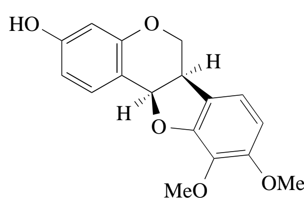
(10) 9-*O*-Methyl coumestrol



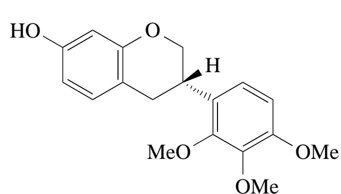
(11) (–)-5'-Methoxysativan



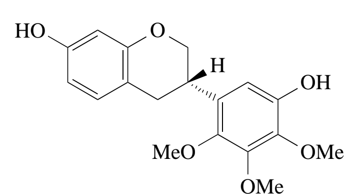
(12) (–)-4-Methoxymedicarpin



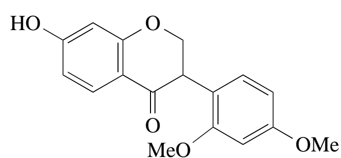
(13) 10-Methoxymedicarpin



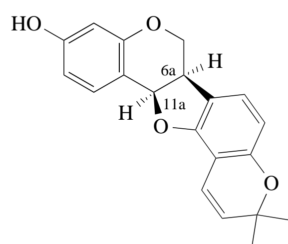
(14) (+)-7-Hydroxy-2',3',4'-trimethoxyisoflavan



(15) (+)-7,5'-Dihydroxy-2',3',4'-trimethoxyisoflavan



(16) Sativanone



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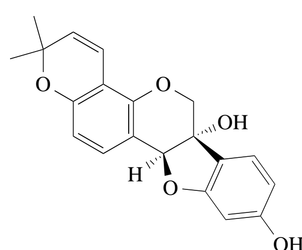
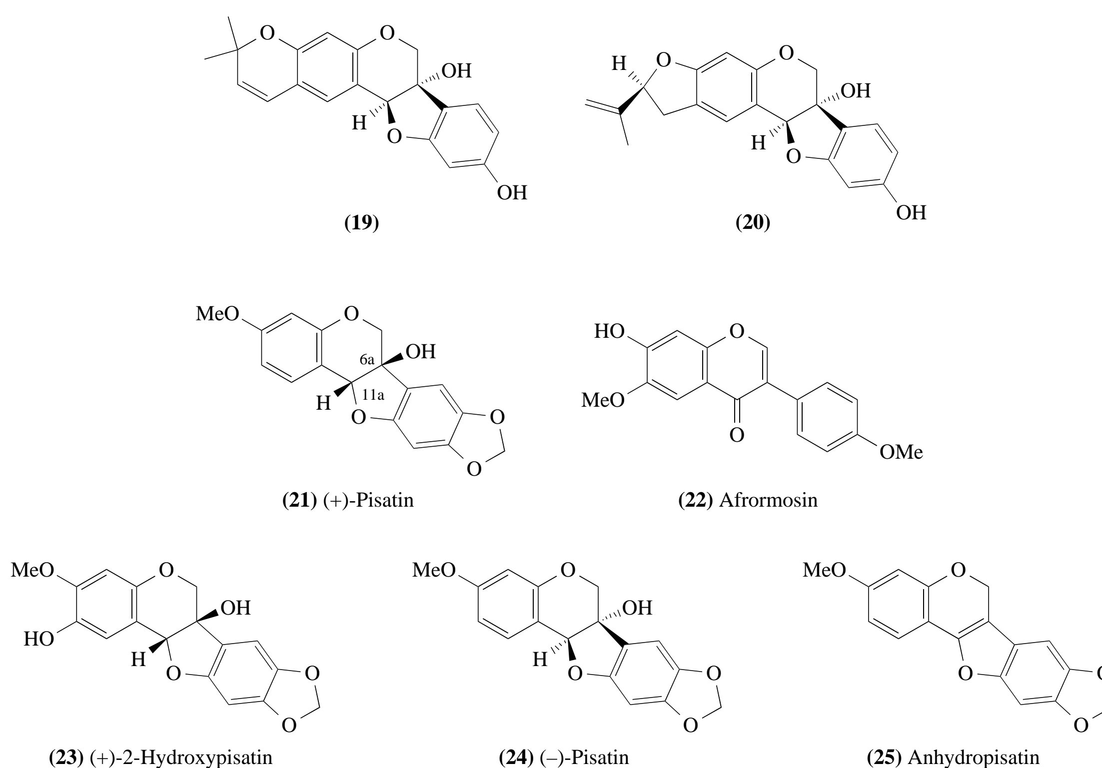


(18)



by Dewick[13]), and the complex santarubin dyes (**30**) that occur along with substituted isoflavenes (**31**) in the red heartwood of the West African tree *Baphia nitida*.[14]

Isoflavonoids have been widely used as taxonomic markers within the Leguminosae. Typical examples include comparative studies among the genera *Medicago* and *Trigonella*.[6,15]

Many excellent reviews have been written on the structure and occurrence of isoflavonoids. The reader is referred to papers by Wong,[16] Dewick,[13,17] Ingham,[18] and Tahara and Ibrahim,[1] and the references cited therein, for detailed information on this topic.

1.28.2 BIOLOGICAL ACTIVITIES OF ISOFLAVONOIDS

1.28.2.1 Overview

The biological activities of isoflavonoids range from properties that suggest important functions in the plant's interaction with its environment to pharmacological properties in animal cells that may or may not reflect corresponding functions/activities in the plant. It is not the purpose of this chapter to review all these biological properties. Many of them are listed in Tables 1 and 2, and only the better studied aspects reviewed in the following section. The multiple roles of isoflavonoids in the relations of plants with their environment have been addressed elsewhere.[39]

1.28.2.2 Role of Isoflavonoids in Plant–Microbial Pathogen Interactions

Isoflavonoids have been ascribed key roles in plant–pathogen interactions because many have strong antimicrobial activity. Antimicrobial isoflavonoids fall into two functional classes, the preformed "phytoanticipins" and the inducible "phytoalexins".[40] Examples of the former class include the prenylated isoflavones of lupin, which are synthesized in various organs of the plant during seedling development.[41] Examples of the latter include several pterocarpans, the biosynthesis of which has been studied in detail, particularly with respect to the phytoalexin response of bean, alfalfa, pea, and soybean (**6, 17–21**).

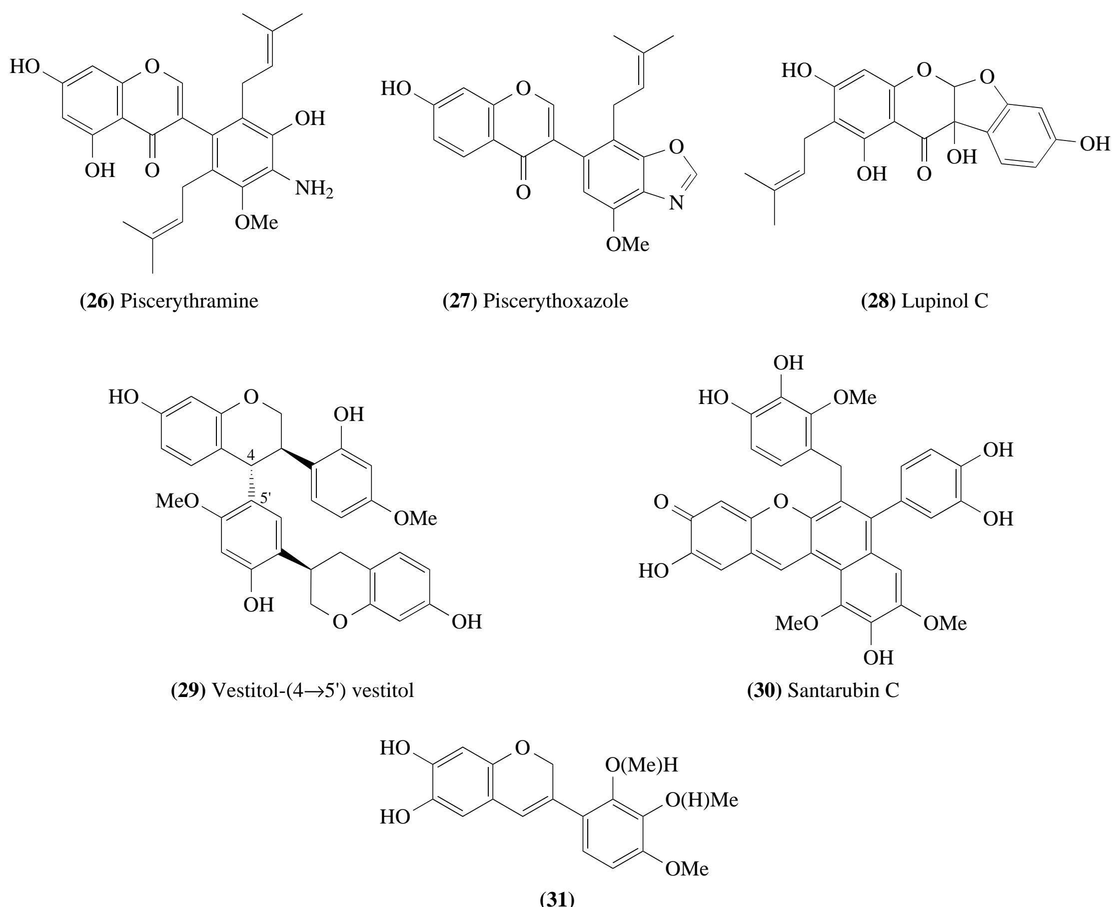


Table 1 Biological activities of isoflavonoids: activities with functional implications for the plant.

Biological activity	*Examples*	*Reference*
Preformed antimicrobial	prenylated isoflavones	see Section 1.28.2.2
Phytoalexin	isoflavans, pterocarpans	see Section 1.28.2.2
Inducer of fungal pathogen spore germination	pisatin (**21**)	see Section 1.28.2.2
Nodulation gene inducer	isoflavones	see Section 1.28.2.4
VAM interaction inducer	various	see Section 1.28.2.3
Nematocidal	glyceollin (**18**)	Huang and Barker[19]
Antiinsect	rotenoids	Fukami and Nakajima[20]
Allelochemical	medicarpin (**6**)	Miller *et al.*[21]
Phytotoxic	phaseollin (**17**), glyceollin (**18**)	Skipp *et al.*[22]; Glazener and VanEtten[23]; Giannini *et al.*[24]
IAA oxidase modulator	lupin isoflavones	Ferrer *et al.*[25]
Control of cell division	sayanedine	Bailey and Francis[26]
Iron chelator	2-(3′,5′-dihydroxyphenyl)-5,6-dihydroxybenzofuran	Masaoka *et al.*[27]
Various pharmacological effects on animals and humans	genistein (**3**), daidzein (**4**), coumestrol (**9**)	see Table 2

There is a vast literature on the isoflavonoid phytoalexins of the Leguminosae. In view of all this research activity, it is surprising that major questions still exist concerning structure–activity relationships and the exact role of these compounds as determinants of disease resistance. Because many plant pathogens have the ability to metabolize, and therefore detoxify, isoflavonoid compounds, structure–activity relations are highly dependent on the fungi used in the bioassays.

Table 2 Biological activities of isoflavonoids: pharmacological activities.

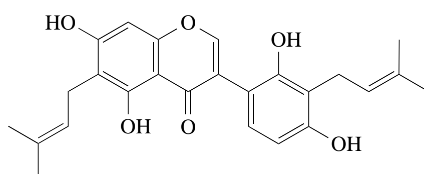
Pharmacological activity	*Compounds*[a]	*Reference*
Mitochondrial ADH inhibitor	daidzin	Keung *et al.*[28]
Antiulcer	G, F, 3′-methoxydaidzein	Takai *et al.*[29]
Antiarthritis	Pseudobaptigenin	Malhotra *et al.*[30]
Estrogenic, proestrogenic	G, F, C	see Section 1.28.2.5
Estrogen receptor binding	C, G, D, F, BA	see Section 1.28.2.5
Antiangiogenic	G	Fotsis *et al.*[31]
Antioxidant	G, many others	Wang and Murphy[32]
Anticancer	G	See Section 1.28.2.5
Protein tyrosine kinase inhibitor (e.g., EGF receptor)	G	Akiyama *et al.*[33]
Protein histidine kinase inhibitor	G	Huang *et al.*[34]
Prostaglandin synthesis inhibitor	isoflavans, isoflavenes	Goda *et al.*[35]
DNA synthesis/cell cycle arrest	G	Takano *et al.*[36]
Topoisomerase inhibitor	G	Okura *et al.*[37]
P_i-purinergic receptor antagonist	G	Okajima *et al.*[38]

[a]G, genistein (**3**); F, formononetin (**5**); 3′-OH-F, 3′-hydroxyformononetin; C, coumestrol (**9**); D, daidzein (**4**); BA, biochanin A (**33**).

A comparison of the effects of a series of isoflavones, isoflavanones, pterocarpans, and isoflavans on the growth of *Aspergillus niger* and *Cladosporium cucumerinum* suggested that lipophilicity and the presence of at least one unsubstitued phenolic hydroxyl group correlated with fungitoxicity.[42] Other studies have suggested that a skewed, aplanar ring structure is essential for high activity of isoflavans, although this has been seriously questioned, and alternative suggestions made that specific combinations of hydrophobic (methoxy) and hydroxyl groups are important, but that no absolute generalizations are possible.[43–45] In the case of the pterocarpan phytoalexins, the stereochemistry associated with the 6a and 11a chiral centers (compare (**17**) and (**21**)) plays an important role in determining antifungal activity, because plant pathogenic fungi are often able to degrade the isomer produced by their host plant but may be highly sensitive to the opposite isomer.[46]

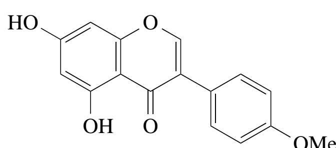
The phytoalexin ''hypothesis'' is based for the most part on indirect determinations of causality, with major reliance on correlative data. Thus, isoflavonoid compounds have been shown to accumulate in infected plant cells to levels shown to be antimicrobial *in vitro*. The temporal, spatial, and quantitative aspects of accumulation are consistent with a role for these compounds in disease resistance.[47–50] However, few studies have directly tested this hypothesis. Inhibition of the synthesis of **(18)** by application of an inhibitor of L-phenylalanine ammonia-lyase (PAL) to soybean seedlings breaks resistance to *Phytophthora megasperma* f. sp. *glycinea*,[51] but this could be due to pleiotropic effects related to down-regulation of the phenylpropanoid pathway as a whole; for example, salicylic acid, a product of the early part of the phenylpropanoid pathway after the PAL reaction, is known to be important for expression of disease resistance.[52] Isolates of *Nectria hematococca* with reduced ability to degrade the pea phytoalexin (**21**) have reduced virulence on pea, suggesting that (**21**) is indeed a factor in the disease resistance response.[53] Ultimately, it will be necessary to produce mutant plants lacking only the isoflavonoid phytoalexins in order to test rigorously the roles of these compounds in plant–microbe interactions. Mutants of *Arabidopsis* lacking the indole phytoalexin camalexin were not less resistant to incompatible bacteria, but did show increased disease symptoms following infection with a compatible race, suggesting that this phytoalexin may play a role in disease symptom limitation rather than in determination of resistance *per se*.[54]

Antifungal isoflavonoids are often as phytotoxic as they are fungitoxic. Compound (**18**) causes proton leakage from *Phytophthora* plasma membrane vesicles and from red beet and soybean tonoplast vesicles.[24] Treatment of bean suspension cells with 30 $\mu g\, mL^{-1}$ of (**17**) resulted in inhibition of respiration within 2 min, and subsequent death of most of the cell population.[22] Pretreatment with lower concentrations of (**17**) does not induce tolerance to higher concentrations, although bean cells do have mechanisms for degrading exogenously added (**17**).[23] These results indicate a requirement for sequestration of bioactive isoflavonoids, be they phytoalexins or phytoanticipins, away from the sensitive molecular sites of the host. In this respect, an immunolocalization study indicated the presence of a diprenylated isoflavone, 2′-hydroxylupalbigenin (**32**) in secondary walls and pericycle cells of lupin roots, and provided evidence for compartmentation in the wall mediated via membrane vesicles.[55]



(32)

Isoflavonoids can act as stimulatory, as well as inhibitory, factors in interactions of legumes with fungi. Daidzein (**4**) and genistein (**3**), important components of soybean root exudates, act at very low concentrations (10 nmol L^{-1}) as chemoattractants for zoospores of *Phytophthora sojae*, and also induce encystment and germination of the zoospores.[56] The isoflavones appear to be inactive with nonpathogens of soybean. Likewise, biochanin A (**33**), (**3**), and several pterocarpan phytoalexins including (**6**) and (**21**) stimulate spore germination of *Fusarium solani* forma speciales pathogenic on pea or bean at a concentration of 10 μmol L^{-1}).[57]



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1.28.2.3 Role of Isoflavonoids in Mycorrhizal Interactions

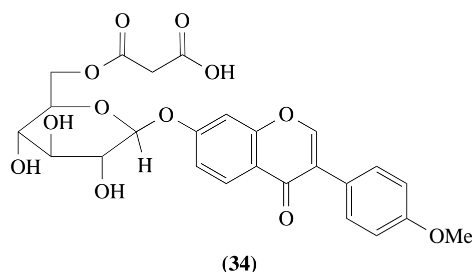
There has been much debate on the possible role of isoflavonoids during the establishment of the symbiotic vesicular arbuscular mycorrhizal (VAM) association of fungi of the species *Glomus* with legume roots. At low concentrations (2–5 μmol L^{-1}), daidzein (**4**) increases the percentage germination of *Glomus* spores by ~35%,[58] and coumestrol (**9**), (**4**), and (**3**) have small but significant stimulatory effects on the degree of mycorrhizal colonization of soybean.[59] It has been suggested that one effect of isoflavonoids on the soybean mycorrhizal symbiosis could be via induction of nodulation (Nod) factors (see below) from cocolonizing *Rhizobia*, since Nod factors have also been shown to stimulate fungal colonization.[59] Once mycorrhizal fungi begin to colonize the host root, an initial increase in isoflavonoid levels is rapidly suppressed.[60,61] The decrease in isoflavonoid levels correlates with a reduction in transcripts of enzymes specific for the later stages of isoflavonoid synthesis throughout the root cortex, although the root cells harboring the fungal arbuscules contain elevated transcript levels for enzymes of the central phenylpropanoid pathway and flavonoid branch pathway, indicating tight and differential control of flavonoid and isoflavonoid synthesis during the establishment and maintenance of this mutually beneficial association.[60,62]

1.28.2.4 Role of Isoflavonoids in the *Rhizobium*–Legume Symbiosis

The establishment of nitrogen-fixing nodules in leguminous plants is initiated by the recognition by the *Rhizobium* bacteria of compounds released in root and seed exudates. Recognition of these compounds (*nod* gene inducers) by the bacterial *NodD* gene products leads to transcription of a set of genes in the bacteria (*nod* genes) which encode biosynthetic enzymes for the formation of substituted lipochitooligosaccharide signal molecules (Nod factors) that in turn induce root hair curling and the cortical cell divisions that characterize the early development of the nodule. Flavonoid and isoflavonoid compounds can play critical roles in these processes. Alfalfa and red clover root exudates contain flavones that potently activate *nod* gene expression,[63,64] whereas the major *nod* gene inducers in the soybean–*Bradyrhizobium* symbiosis are the isoflavones daidzein (**4**) and genistein (**3**).[65] Reduced synthesis of (**3**) in roots at suboptimal temperatures may represent a limitation to Rhizobial colonization.[66]

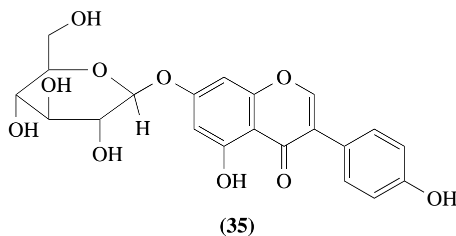
Root exudates from alfalfa plants inoculated with *Rhizobium meliloti* contain the pterocarpan medicarpin (**6**) and its glucoside, as well as formononetin 7-*O*-glucoside-6″-*O*-malonate (FGM) (**34**). Levels of (**34**) are increased when plants are grown under low nitrogen conditions.[67] Formononetin (**5**) and its 7-*O*-glucoside do not possess *nod* gene-inducing activity for the alfalfa symbiont. Sur-

prisingly, however, (**34**) can induce *Rhizobium nod* genes through interactions with both the NodD1 and NodD2 recognition proteins.[68] The Nod factors synthesized as a result of *nod* gene activation are active on alfalfa roots at concentrations of around 10^{-9} mol L^{-1}. At higher concentrations (10^{-6} mol L^{-1}), they have been shown to induce genes of the isoflavonoid biosynthetic pathway in microcallus cultures,[69] but it is not clear whether this represents a physiologically relevant defensive response by the plant.



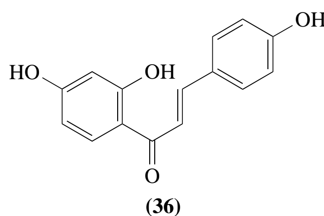
(34)

Following inoculation of bean (*Phaseolus vulgaris*) roots with *Rhizobium leguminosarum*, coumestrol (**9**), (**4**), (**3**), and genistin (**35**) are released in the root exudate. Of these compounds, (**9**) and (**4**) activate transcription under control of the *R. leguminosarum nodD*1 gene.[70]



(35)

Compounds (**3**), (**4**), and (**9**) produced by soybean are inactive as chemoattractants for *Bradyrhizobium japonicum*, whereas hydroxycinnamic acid precursors are strong attractants.[71] The chalcone precursor of (**4**), isoliquiritigenin (**36**), is an order of magnitude more potent than (**4**) as a *nod* gene inducer, but is not a chemoattractant.[72]



(36)

It has been suggested that internal isoflavonoids may also play a role in later stages of nodulation in soybean, because their levels are elevated in hypernodulating mutants, and nitrogen application reduces isoflavonoid levels in parallel with decreased nodule number, weight, and nitrogenase activity.[73] Increased nodule numbers in a hypernodulating mutant are not observed until $\sim$9 d after initial bacterial inoculation.[74] Reciprocal grafting experiments between a wild-type and a hypernodulating soybean mutant have shown that root isoflavonoid levels are controlled by the shoot.[74] Treatment of soybean roots with abscisic acid (ABA) leads to reduction in both nodulation and isoflavonoid levels, although a comparison of wild-type, hyper-, and hyponodulating mutants did not reveal significant differences in endogenous ABA levels, suggesting that ABA is not the factor produced by the shoots to autoregulate nodulation.

Levels of glyceollin I (**18**) increase by a factor of 50 in soybean root exudates following inoculation with *B. japonicum*, and increases are also observed in the levels of the *nod* gene inducers (**4**), (**3**), and (**9**).[75,76] The induction of (**18**) does not require bacterial penetration, as it can be mimicked with heat-killed cells.[75] The levels of (**18**) obtained are, however, significantly lower than those observed in the response of soybean roots to an incompatible race of the fungal pathogen *Phytophthora megasperma* f. sp. *glycinea*. Levels of (**4**), (**3**), and (**9**) are not elevated in root exudates following

inoculation with mutant *B. japonicum* that cannot produce Nod factors, and pure nonsulfated Nod factor (as produced by *B. japonicum*) itself can induce these isoflavonoids.[76]

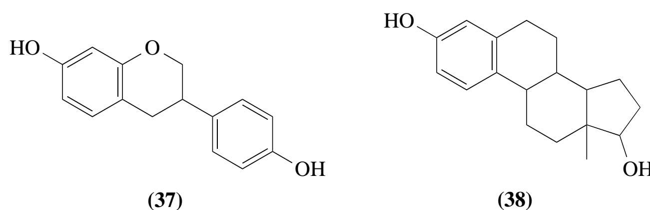
B. japonicum is sensitive to (**18**), but can tolerate it following adaptation to low concentrations. This resistance, which does not involve detoxification or degradation, can also be induced by (**4**) and (**3**), and is not dependent on NodD, the protein that binds isoflavonoids to induce *nod* genes.[77] Therefore, isoflavonoids act at different sites to induce glyceollin tolerance and *nod* gene activity. Induced glyceollin tolerance may be important for survival of *B. japonicum* in the rhizosphere.

It appears that isoflavonoids can induce rhizobial genes in addition to those involved in production of Nod factors.[78] Further studies are necessary to confirm whether isoflavonoid turnover and isoflavonoid-induced gene expression are important features of the regulatory cross-talk between host and symbiont.

1.28.2.5 Effects of Isoflavonoids on Animal and Human Health

It has been known for many years that dietary isoflavonoids can exert estrogenic effects in animals. For example, reports as early as 1946 documented the occurrence of infertility in sheep resulting from grazing on clover rich in the isoflavone formononetin (**5**),[79] and breeding programs have been devised to select for low isoflavone lines of subterranean clover.[80] It has been suggested that California quails might control their natural populations during periods of low food supply by feeding on legumes rich in daidzein (**4**), genistein (**3**), and/or coumestrol (**9**).[81]

Compounds (**4**), (**3**), and equol (**37**) (a major metabolite of dietary isoflavonoids formed by the gastrointestinal flora) share structural features with the potent estrogen estradiol-17β (**38**), particularly the phenolic ring and the distance (11.5 Å) between the two hydroxyl groups, features that determine ability to bind estrogen receptors. Isoflavonoids can thus exert both estrogenic and antiestrogenic activity, the latter by competing for receptor binding by (**38**). However, isoflavonoids and their gastrointestinal metabolites have relatively weak estrogenic activity.[82,83] Compounds (**37**) and (**3**) are active in displacing bound estrogen and testosterone from human sex steroid binding protein,[84] suggesting that phytoestrogens might also affect clearance rates of androgens and estrogens and thus the availability of the hormones to target cells.



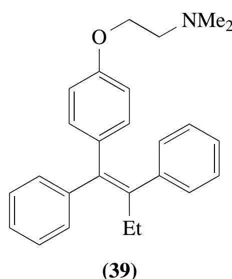
(37) **(38)**

Compound (**5**) has been shown to stimulate mammary gland proliferation and to increase estrogen receptor and plasma prolactin levels in mice.[83] Although these effects mimic the action of (**38**), (**5**) is 15 000 times less potent than (**38**) for binding to murine mammary estrogen receptors.[83] Hence its major biological activity is probably as an estrogen agonist, although its concentration in the "normal Western" diet is probably too low to have any physiological effects. However, in humans eating a soy protein-rich diet, isoflavonoids may be present in the urine at very high levels. Thus, human adults given a diet containing 40 g of soy protein per day secreted 5.3 mg of (**37**) per day, compared with only 2–27 μg of the principal urinary estrogen, estrone glucuronide, released during the follicular phase of the menstrual cycle.[82] This is a 100-fold increase in urinary (**37**) above that observed in adults who consume very little soy products in their diet.

The major interest in dietary isoflavonoid phytoestrogens is because of the significant correlations demonstrated between a soy-rich diet and reduced incidence of breast cancer or mortality from prostate cancer. The incidence of breast cancer is 5–8-fold lower among women from Japan who consume a traditional diet than among women in the United States or Europe who consume a diet higher in animal fat but with very little soy products.[85] A detailed epidemiological study of Singapore Chinese women (420 healthy controls and 200 with histologically confirmed breast cancer), indicated that soy consumption was directly correlated with reduced risk of cancer.[86] The offspring of oriental women who have emigrated to the United States have the same risk of breast cancer as American women if they adopt the Western diet, suggesting that oriental women are not simply genetically predisposed against breast cancer.

Feeding rats a diet containing powdered soybean chips strongly reduced mammary tumor formation in response to the direct carcinogen *N*-methyl-*N*-nitrosourea, with no effect on estrus cycling.[85] The soybean diet resulted in elevated levels of hepatic PAPS:sulfotransferase activity, which may be involved in deactivating metabolically activated carcinogens. It was concluded that the effects of the soybean diet could be the result of estrogenic substances acting as inhibitors of estrogen action or, because similar reduction in tumor formation was observed in response to the procarcinogen 7,12-dimethylbenz[*a*]anthracene, as inducers of hepatic metabolism.

Urinary excretion of (**4**), (**3**), and (**37**) was shown to be at least 10-fold higher in a population of farm workers from Japan compared with Americans or Europeans, and it was suggested that the isoflavonoids found in soy products might be the agents responsible for reduced cancer risk.[87] In fermented soybean foods, the isoflavonoids are usually present as the aglycones, whereas the β-glycosides predominate in nonfermented products.[88] A 1 g amount of powdered soybean chips contains nearly 800 μg of (**4**) and >500 μg of (**3**), whereas 1 g of soya protein has ~ 150 μg of (**4**) and 250 μg of (**3**).[85] Structural similarities have been noted between soybean isoflavones and tamoxifen (**39**), an antiestrogen which has been clinically tested as a chemopreventive agent in women with high risk of breast cancer.[88]



(39)

When administered neonatally, (**3**) effectively protects against chemically induced mammary tumors in rats.[89] The effects include increased latency, reduced tumor incidence and multiplicity, and more rapid maturation of undifferentiated end buds to differentiated lobules. Although no clinical trials have been reported documenting effects of controlled dietary supplementation with (**3**) on breast cancer incidence in humans, it has been shown that a high soy diet containing up to 45 mg of isoflavones per day causes changes in the menstrual cycle that may help reduce cancer risk.[90] It has been suggested that the high levels of isoflavones in breast milk of humans consuming a high soy diet may provide the infant with protection against cancer later in life.[91]

Compounds (**3**) and (**33**) inhibit the growth of human stomach cancer cell lines *in vitro*, apparently by stimulating a signal transduction pathway leading to apoptosis.[92] When these cancer cells were transplanted into mice, (**33**), but not (**3**), significantly inhibited tumor growth.

Compound (**3**) can affect a number of molecular processes, one or more of which may be associated with its pharmacological effects. Thus, in addition to showing estrogenic properties in receptor binding and whole tissue studies, (**3**) is an inhibitor of several enzymes, including DNA topoisomerase and tyrosine protein kinase,[33] and also exhibits antioxidant properties and cell cycle arrest activity. Kinase inhibition is generally regarded as being specific for tyrosine kinases such as epidermal growth factor receptor, $pp60^{v\text{-}src}$ and $pp110^{gag\text{-}fes}$, although at higher concentrations (**3**) also inhibits protein histidine kinase.[34] Compound (**3**) blocks EGF-mediated tyrosine phosphorylation *in vivo* in human epidermal carcinoma cells.[33] However, as (**3**) does not block epidermal growth factor phosphorylation *in vivo* at a concentration that reduces mammary tumor formation, it is unlikely that its chemopreventive activity is a result of its activity as a protein kinase inhibitor.[93] Nevertheless, when specifically targeted to the B-cell-specific receptor CD-19 by conjugation to a monoclonal antibody, (**3**) selectively inhibited CD-19-associated tyrosine kinase activities, resulting in death of human B-cell precursor leukemia cells.[94] Other isoflavones such as (**4**) do not inhibit tyrosine kinase activity, and are therefore used as controls in pharmacological experiments utilizing (**3**).

Compound (**3**) potently, specifically, and directly inhibits glucose and dehydroascorbate uptake by the mammalian facilitative hexose transporter GLUT 1.[95] It also inhibits fast sodium channels in human uterine leiomyosarcoma cells, a process that is also inhibited by (**4**) and is therefore independent of tyrosine kinase activity.[96] Unlike other isoflavonoids, (**3**) only exerts toxicity at concentrations greatly in excess of those at which it first exerts its biological effects, making it an important subject for future studies on cancer chemoprevention. Further information on the clinical

effects of isoflavonoid phytoestrogens can be found in reviews by Adlercreutz *et al.*,[97] Messina *et al.*,[98] Knight and Eden,[99] and Wiseman.[100]

1.28.3 BIOSYNTHESIS OF ISOFLAVONOIDS

1.28.3.1 Experimental Systems for the Study of Isoflavonoid Biosynthesis

As described above, isoflavonoids may be either constitutively synthesized under the control of developmental programs, and/or induced in response to environmental cues such as pathogen infection. Tissues in which isoflavonoids are made constitutively have been less popular as model systems for biosynthetic studies than have inducible systems. Exceptions include cases where the constitutive isoflavonoids have particular substitutions of interest, such as the prenylated isoflavonoids of *Lupinus* and several other species,[1] or where a very wide range of isoflavonoids occurs in plants with medicinal value, such as in Kudzu vine (*Pueraria lobata*).[101]

Elicitor-treated plants and cell suspension cultures have been widely used for studies of isoflavonoid biosynthesis, enzymology, and molecular biology. The first studies to define the basic pathways leading to pterocarpanoid compounds utilized seedlings exposed to $CuCl_2$ through the roots.[102,103] This toxic abiotic elicitor induces the synthesis of most of the isoflavonoids encountered in infected tissues. Improved exposure of cells to both elicitor and labeled precursors, and simplicity of metabolite extraction due to lack of interfering chlorophyll, are features that have made elicitor-treated cell suspension cultures the most popular system for isoflavonoid biosynthetic studies.[104] Such cultures also provide large amounts of elicited material for enzyme purification. Heavy metal ions are often poor elicitors in cell suspension systems owing to their overall toxicity to the culture. Preferred elicitors are preparations, usually containing glycans and glycoproteins, obtained from the cell walls of phytopathogenic fungi or from yeast. The nature and properties of such biotic elicitors have been extensively reviewed.[105,106] The tripeptide glutathione has been used as a cheap, convenient, and biologically reproducible elicitor with some cell and organ culture systems, although it is inactive with others.[107,108]

The response of cell cultures to exogenously applied elicitors is dependent upon the growth stage of the culture, the exact nature of the culture medium (particularly the plant growth regulators and their levels), and the nature and concentration of elicitor applied. The systems for which these various parameters have been most studied are bean, soybean, alfalfa, and chickpea cell suspension cultures. Table 3 outlines the features of a range of cell and organ culture systems that have provided important information on isoflavonoid biosynthesis and its control. The references cited point the reader to the conditions for establishment and use of the culture system.

Although cell culture systems have obvious advantages for basic biochemical and molecular studies, they lack the spatial organization of the intact plant. Isoflavonoid accumulation may vary quantitatively and qualitatively depending on the distance from the applied stimulus. Such distinct proximal and distal cell responses have been best characterized in soybean using an elicitor-treated cotyledon system.[113,114]

In the intact plant, the rate and extent of production of isoflavonoid phytoalexins in response to microbial pathogens depend on the genotype of the host and the particular race of the pathogen.[115] This so-called race-specific or gene-for-gene resistance, which is often determined by single complementary genes in both host and pathogen, is usually not easy to mimic in a cell culture system, as most elicitors that have been isolated do not share the specificity for a particular host genotype that is characteristic of the organism from which they were isolated. A soybean cell culture system has been developed in which gene-for-gene resistance is expressed in response to the bacterial pathogen *Pseudomonas syringae* pv *glycinea*,[116,117] and such systems will be of value for future studies on the molecular genetic control of isoflavonoid synthesis in response to specific host resistance gene-mediated signaling pathways.

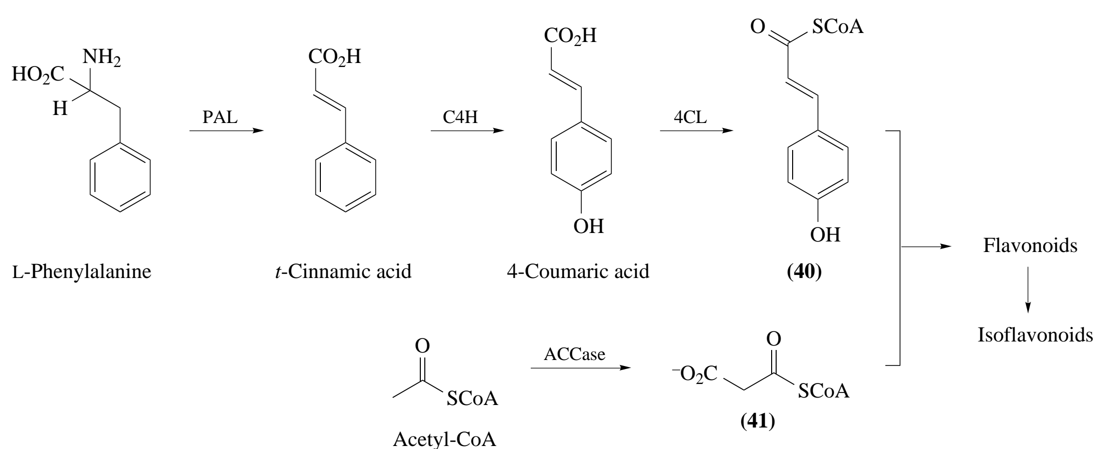
1.28.3.2 Role of the Central Phenylpropanoid and Acetate–Polymalonate Pathways in Isoflavonoid Synthesis

The B-ring of the C_{15} skeleton of flavonoids and isoflavonoids originates from the phenylpropane unit of 4-coumaroyl-CoA (**40**), whereas the A-ring is derived from head-to-tail condensation of three molecules of malonyl CoA (**41**), derived from acetyl-CoA (Scheme 2). In elicitor-treated cell

Table 3 Plant cell and organ culture systems used for the study of isoflavonoid biosynthesis.

Species	*Culture*	*Elicitor*	*Compounds*	*Ref.*
Cicer arietinum (chickpea)	cell suspension cultures (resistant and susceptible to *Ascochyta rabei*)	from *A. rabei*; yeast elicitor	pterocarpans	109
Glycine max (soybean)	cell suspension cultures	from *Phytophthora megasperma* f. sp. *glycinea*	prenylated pterocarpans	110
Lotus corniculatus (birdsfoot trefoil)	*Agrobacterium rhizogenes*—transformed hairy root cultures	from *Rhyncosporium orthosporum*; glutathione	isoflavans	107
Lupinus polyphyllus, *Lupinus hartwegii*	*Agrobacterium rhizogenes*—transformed hairy root and suspension cultures	not used	isoflavone glycosides	111
Medicago sativa (alfalfa)	cell suspension cultures	from *Colletotrichum lindemuthianum*; yeast elicitor	pterocarpans	112
Phaseolus vulgaris (French bean)	cell suspension cultures	from *Colletotrichum lindemuthianum*; yeast elicitor	prenylated pterocarpans	104
Pueraria lobata (Kudzu vine)	cell suspension cultures	yeast elicitor	isoflavones and isoflavone dimers	101

cultures, the enzymes of the core phenylpropanoid pathway, L-phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumarate:CoA ligase (4CL), as well as the acetyl-CoA carboxylase (ACCase), are coinduced with the later enzymes specific for isoflavonoid synthesis.[118,119] Indeed, elicitor-treated cell cultures provided the biological material for cloning of the cDNAs encoding these four elicitor-induced enzymes,[119–122] and many papers have described the induction of PAL and 4CL at the enzyme activity, protein, and transcript levels in relation to isoflavonoid synthesis (reviewed by Dixon and Harrison[123]).



Scheme 2

PAL is encoded by a family of at least three genes in most species studied. In bean cell cultures, elicitation leads to the preferential appearance of the PAL isoenzymes with the highest affinity (lowest K_m value) for phenylalanine, prior to the accumulation of phaseollin (**17**).[124] Whether this

reflects a mechanism for the specific channeling of phenylalanine into isoflavonoid synthesis, or whether it simply increases the overall flux into the phenylpropanoid pathway, remains to be determined. It is also not clear whether specific isoforms of 4CL play a regulatory role in isoflavonoid synthesis.

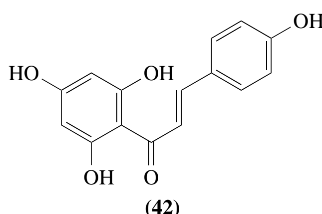
Studies on transgenic tobacco plants that overexpress or underexpress PAL have demonstrated that, under normal conditions, PAL is the rate-limiting step for the synthesis of hydroxycinnamic acid esters such as chlorogenic acid, but not for the flavonoid rutin.[125,126] It is therefore likely that the rate-determining steps for isoflavonoid synthesis in legumes are downstream of the enzymes of the core phenylpropanoid pathway, although the enzymes may have to be induced to accommodate the increased flux following elicitation. The acetyl-CoA carboxylase cDNA cloned from elicited alfalfa cell cultures encodes a cytoplasmic form of the enzyme that could be involved in both fatty acid elongation and flavonoid/isoflavonoid synthesis.[119] Demonstration of whether the carboxylase activity is rate limiting for flavonoid/isoflavonoid synthesis will require the generation of transgenic plants with altered activity levels of this enzyme.

1.28.3.3 Reactions of Isoflavonoid Biosynthesis as Determined by Radiotracer Experiments

A series of pioneering experiments by Dewick and collaborators between 1978 and 1983 helped define the sequence of individual reactions leading to the formation of isoflavones, isoflavans, and pterocarpans.[102,103,127–131] These studies measured incorporation of radiolabeled precursors in $CuCl_2$-treated seedlings of alfalfa, bean, pea, and red clover. The conclusions were essentially supported by results of parallel studies in which many of the proposed biosynthetic intermediates were isolated and characterized.[132,133]

The pathways as they are currently understood are shown in Scheme 3, which outlines several clear principles for the elaboration of isoflavonoid structures. First, the isoflavone daidzein (**4**) is the first isoflavonoid product, having been formed by the isoflavone synthase reaction. It should be noted that genistein (**3**) is the corresponding precursor for a series of isoflavonoids that retain the 5-hydroxyl group that originates as the 6′-hydroxyl of 2′,4,4′,6′-tetrahydroxychalcone (naringenin chalcone) (**42**), the normal product of the chalcone synthase reaction that is formed in the absence of chalcone reductase (see below). Pterocarpans and isoflavans are then formed following 2′-hydroxylation of the isoflavone and reduction to the corresponding isoflavanone. Some substitutions of the B-ring, such as *O*-methylation, further hydroxylation, and methylenedioxy ring formation, occur prior to reduction to 2′-hydroxyisoflavanone.[102,103,113,114]

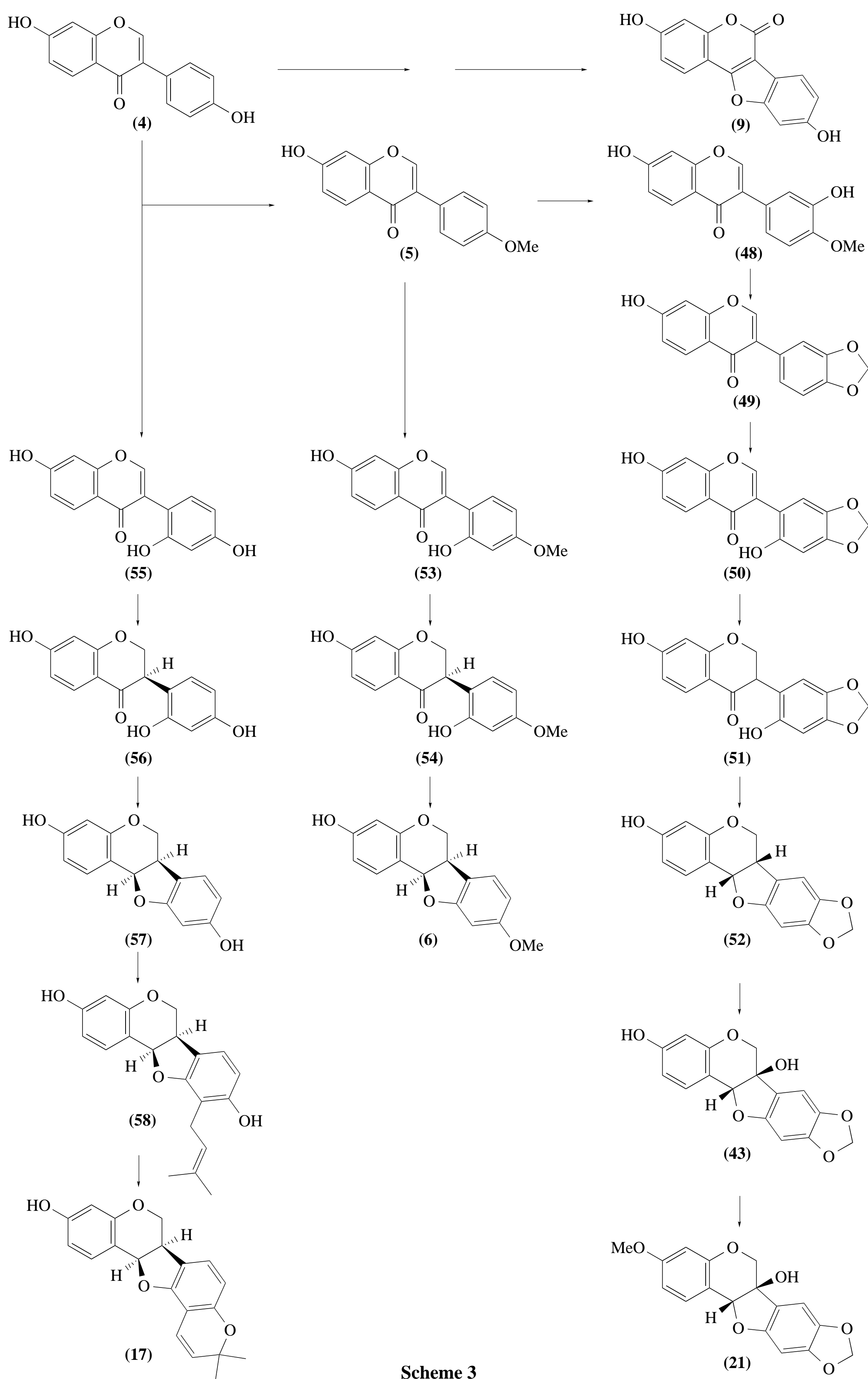
Further substitutions such as prenylation or 6a-hydroxylation occur after ring closure to yield the pterocarpan nucleus.[127,129] The final reaction of pisatin (**21**) biosynthesis is the A-ring *O*-methylation of (+)-6a-hydroxymaackiain (**43**).[129]



(42)

The above labeling experiments demonstrated that medicarpin (**6**) and vestitol (**8**) were interconvertible in alfalfa, and this was explained on the basis of a common carbonium ion intermediate (**44**), formed from an isoflavanol (**45**), that could be involved in the synthesis of pterocarpans, isoflavans, and possibly also coumestans[103] (Scheme 4). On the basis of radiotracer experiments, coumestans were proposed to arise via isoflav-3-enes (**46**) and 3-arylcoumarins (**47**) [131] (Scheme 4). One apparently contradictory result to arise from these studies was the unexpected lack of incorporation of (**4**) into (**6**) (via formononetin (**5**)) in alfalfa, although this compound was a good precursor of coumestrol (**9**). The possible mechanism of isoflavone 4′-*O*-methylation is discussed in detail in Section 1.28.3.10.1.

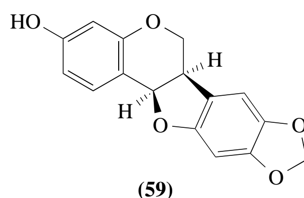
The isoflavanones and pterocarpans have one and two chiral centers, respectively, and the enzymes involved in their biosynthesis exhibit strict stereochemical requirements. In most species that have been described, the pterocarpans are of the (−)-configuration, although (+)-pisatin (**21**) occurs as the major phytoalexin in pea. Labeling studies with enatiomeric precursors indicated the preferential incorporation of (+)-(6a*S*, 11a*S*)-maackiain (**52**) over (−)-(6a*R*, 11a*R*)-maackiain (**59**) into (+)-



Scheme 3

Scheme 4

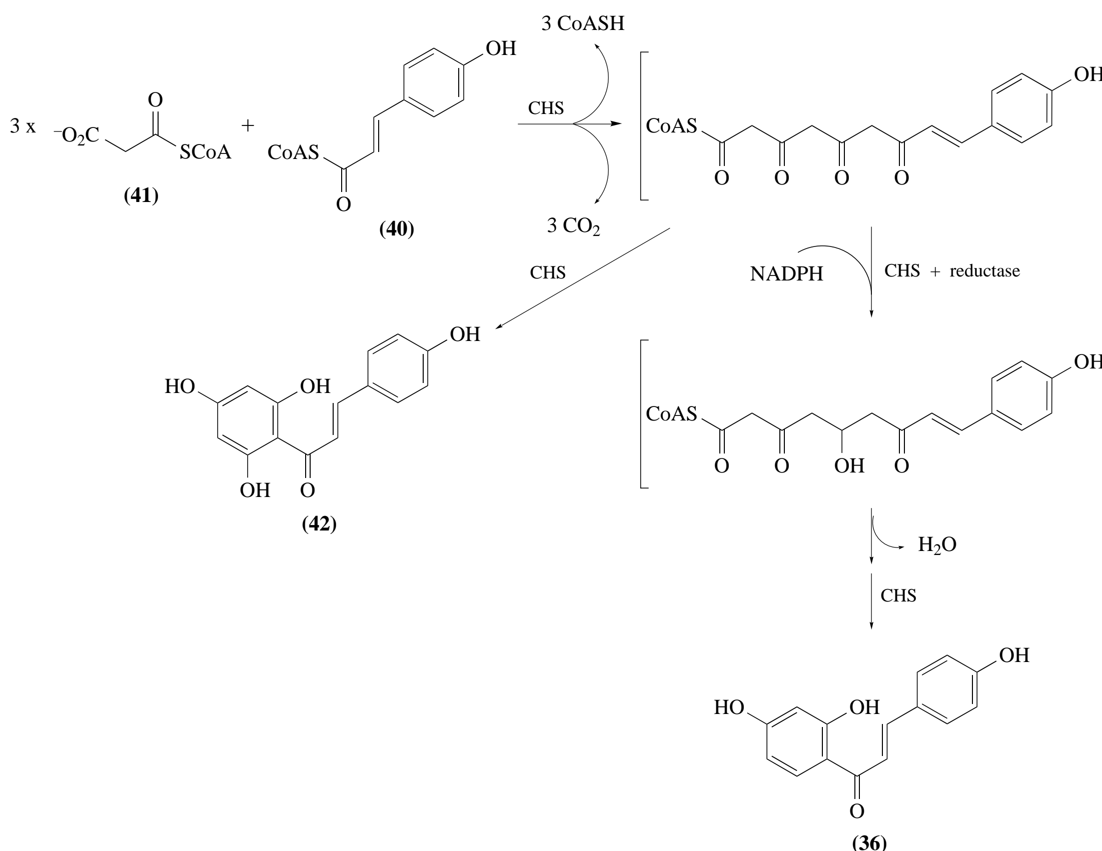
(6a*R*, 11a*R*)-pisatin (**21**) (note that assignment of *S* and *R* configuration is changed following hydroxylation of the 6a position), establishing that the 6a-hydroxylation of pterocarpans occurs with retention of configuration.[128] This would be predicted because inversion would also require additional inversion at C-11a as pterocarpans have a Z-fused ring system. A full understanding of the chemical and enzymological basis of pterocarpan stereochemistry is of significant importance for attempts to improve phytoalexin efficiency through genetic engineering, because plant pathogens are often unable to metabolize the opposite stereoisomer to that produced in their host plant(s).



(59)

1.28.3.4 Chalcone Synthase and Chalcone Reduction

The first C_{15} precursor of the isoflavonoids is the chalcone derived from the head-to-tail condensation of 4-coumaroyl-CoA (**40**) and three molecules of malonyl CoA (**41**) catalyzed by the enzyme chalcone synthase (CHS). CHS is a dimeric polyketide synthase, subunit $M_r \approx 42\,000$, which catalyzes the addition, condensation, and cyclization reactions leading to the formation of 2′,4,4′,6′-tetrahydroxychalcone (naringenin chalcone) (**42**) (Scheme 5). CHS has been purified and characterized, and its genes cloned, from many plant species.[134–138] Further discussions here will be limited to aspects of its action and expression specifically related to its participation in the synthesis of isoflavonoids.



Scheme 5

The genetic model plant *Arabidopsis thaliana* contains a single *CHS* gene,[139] which is clearly sufficient for the basic functions of plant growth and development. However, in most legume species, CHS is encoded by multigene families, consisting of 6–8 members in green bean (*P. vulgaris*),[140] at least seven in soybean,[135] at least seven in pea,[136] at least four in subterranean clover,[137] six or seven in *Pueraria lobata*,[141] and more than seven in alfalfa.[138] Gene family members are often tightly clustered in the genome,[135,137,140] suggesting that they have arisen from fairly recent gene duplication events. It has been suggested that the multiple forms of CHS in legumes may have evolved to serve particular specializations of the flavonoid pathway, for production of isoflavonoid phytoalexins and flavonoid/isoflavonoid/chalcone nodulation gene inducers. However, there is currently no direct evidence in support of this hypothesis. In alfalfa, at least five different members of the *CHS* gene family are constitutively expressed in roots and root nodules, but not in the aerial parts of the plant. However, these family members are expressed in leaves, at the onset of the isoflavonoid phytoalexin defense response, following exposure to pathogens or elicitors.[138] The CHS proteins encoded by the different gene family members are generally very similar in primary sequence, and it is not known if they possess different kinetic properties or are differentially localized in the cell.

Induction of CHS at the level of activity, protein, transcript levels, translatable mRNA activity, or transcription rate has been demonstrated in cells of many legume species in relation to elicitation of isoflavonoids.[138,142–145] Considerable attention has been paid to the regulatory mechanisms

whereby *CHS* genes are activated in response to developmental and environmental cues, and promoter elements and their cognate transcription factors involved in the switching on of expression of the gene during the isoflavonoid phytoalexin response have been identified.[146]

Many isoflavonoids lack the 5-hydroxyl group (6′-hydroxyl, chalcone numbering), and are derived from 2′,4,4′-trihydroxychalcone (**36**) rather than from (**42**). The 5-deoxyisoflavonoids are particularly prevalent in legume roots, and the pterocarpan phytoalexins are invariably of this class. ^{13}C-labeling studies indicated that the 5-hydroxyl group was lost prior to the cyclization of the A-ring of the chalcone,[147] presumably at the polyketide stage. After many unsuccessful attempts to demonstrate the reaction *in vitro*, it was shown that a crude extract from elicited cell cultures of *Glycyrrhiza echinata* could produce (**36**) and its corresponding flavanone liquiritigenin (**60**), in addition to naringenin (**61**), from (**40**) and (**41**) in the presence of high concentrations of NADPH.[148] Compound (**36**) was produced first, and then converted to (**60**) by chalcone isomerase present in the preparation. The activity was described as 6′-deoxychalcone synthase, and was also demonstrated in *G. echinata* protoplasts.[148]

The mechanism of 6′-deoxychalcone formation became apparent when it was shown that purified soybean CHS required the presence of a separate protein, given the trivial name "chalcone reductase" (CHR), for NADPH-dependent formation of (**36**).[149] The reductase was purified to apparent homogeneity, and was shown to be a monomer, of M_r 34 000, that catalyzed the transfer of the pro-*R*-hydrogen of [4-^{3}H]NADPH to the polyketide bound to CHS, with resultant loss of the hydroxyl function as water (Scheme 5). The enzyme had a pH optimum of 6.0, a K_m for NADPH of 17 μmol L^{-1}, exhibited approximately 90% maximum activity at a molar ratio (CHS:reductase) of 2:1, and could coact with CHS from parsley, a species that does not synthesize 6′-deoxychalcone derivatives.[149] This latter point suggests that the multiple forms of CHS found in legumes are unlikely to be involved differentially in the formation of 6′-deoxy and 6′-hydroxychalcones.

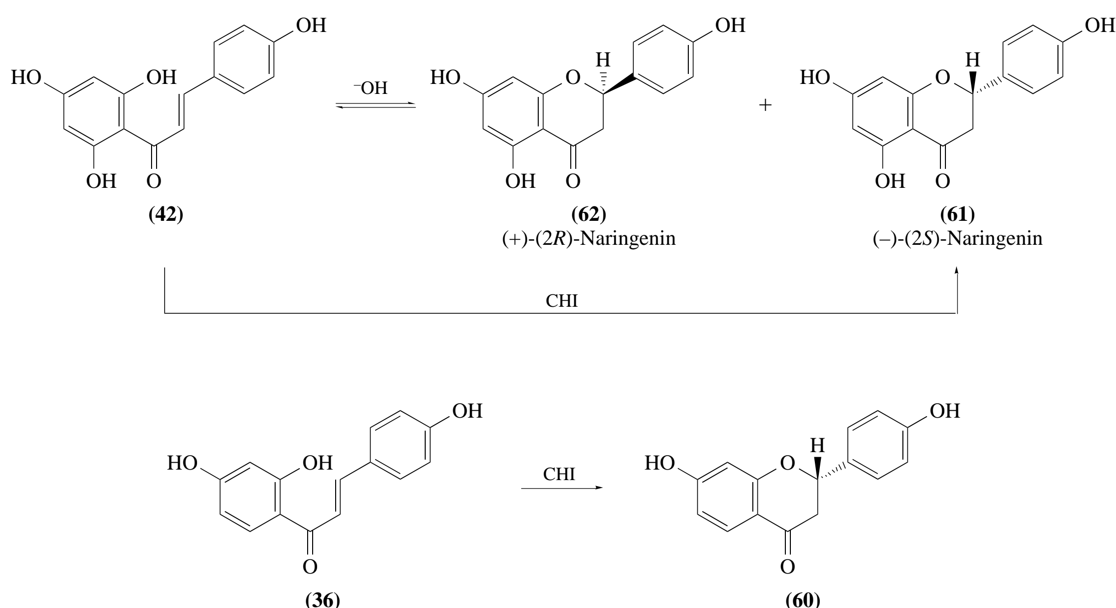
Antibodies were raised against the soybean reductase[150] and cDNA clones were obtained.[151] CHR is encoded by a small gene family in soybean[151] and alfalfa,[152–155] and has also been cloned from *Pueraria lobata* and *Glycyrrhiza echinata*;[101,155] the gene does not appear to be present in species such as carrot and parsley that do not accumulate 5-deoxyisoflavonoids.[151] CHR can be functionally expressed in *Escherichia coli*[151] and recombinant enzyme can be obtained in milligram quantities from this source.[156] The enzyme possesses a leucine zipper domain, but it is not known if this is involved in interactions with CHS. Although the enzyme is a polyketide reductase, it does not share significant sequence identity to the reductases of fatty acid synthesis; rather, it is related to a mammalian aldose reductase and prostaglandin synthase, and to 2,5-diketo-D-gluconic acid reductase from *Corynebacterium*.[151] It is still not clear why coaction of CHR with CHS never results in more than 50% formation of the 6′-deoxychalcone. This is the case with the enzyme if purified from plant sources or if produced in *E. coli*. Interaction of recombinant CHR with a CHS heterodimer containing a single active site produced no significant difference in 6′-deoxy to 6′-hydroxyl product ratio from that observed with wild-type CHS, indicating that the production of both chalcones cannot result from the presence of two functionally distinct active sites (i.e., one coupled to CHR and one not).[157]

Studies using CHR antibodies and cDNA probes have demonstrated closely coordinated induction of CHR with CHS at the protein, mRNA activity or transcription rate levels in elicited cell suspension cultures of soybean,[150] *G. echinata*,[158] and alfalfa.[159]

1.28.3.5 Chalcone Isomerase. Formation of the Immediate Precursor for Isoflavone Formation

At alkaline pH, naringenin chalcone (**42**) isomerizes spontaneously to the corresponding flavanone, naringenin, yielding a racemic mixture of (+)- and (−)-forms (**61,62**) (Scheme 6). This reaction occurs less readily with (**36**). The substrate for aryl migration to isoflavone is the (−)-(2*S*)-flavanone (see below), and it is formed *in planta* from the corresponding chalcone by the activity of chalcone isomerase (CHI). CHI has been purified from many sources,[160–162] and cDNAs and genomic clones have been characterized.[163–168] The enzyme is generally present at significantly higher activity levels than CHS, but is nevertheless induced by elicitor treatment in various legume cell suspension cultures at the onset of isoflavonoid accumulation.[169,170]

CHI catalyzes a net intramolecular *cis*-addition to the chalcone double bond (Scheme 6), and various models have been proposed to describe the stereochemical course of the reaction.[171,172] The size of the enzyme appears to vary depending on its source. In legumes such as bean and alfalfa, it has an M_r of ~28 000 and, in contrast to CHS, appears to exist as a single form.[173] The CHIs from

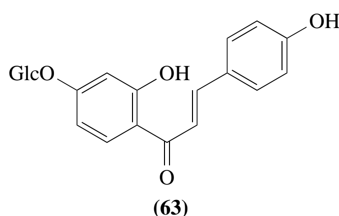


Scheme 6

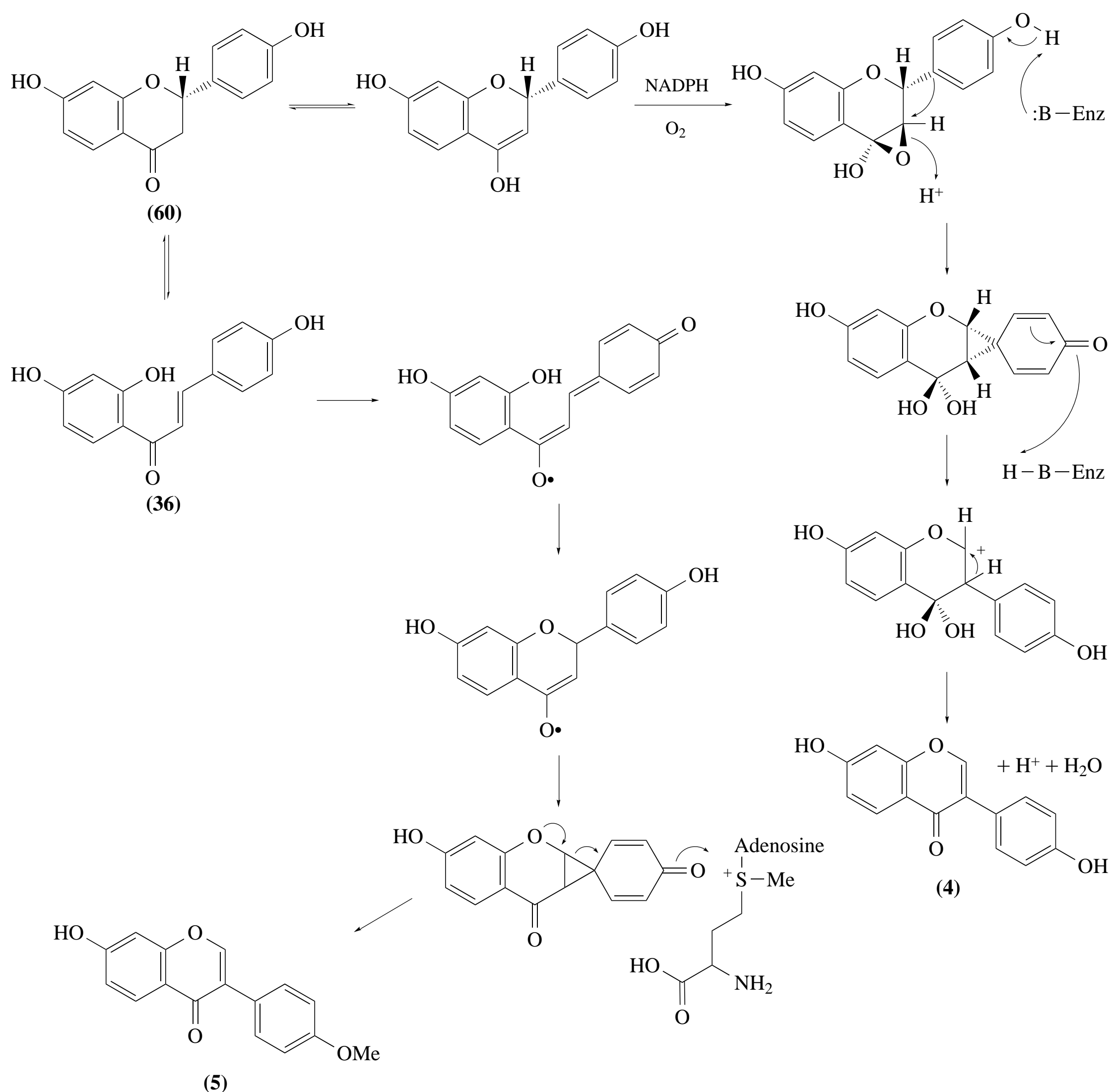
legumes catalyze the isomerization of both (**36**) and (**42**), whereas the former is not utilized by the enzyme from parsley, *Petunia*, *Dianthus*, or *Callistephus*, plants that do not make the 5-deoxy class of flavonoids/isoflavonoids.[173]

1.28.3.6 "Isoflavone Synthase." The First Committed Step of the Isoflavonoid Pathway

The chalcone/flavanone pair represents the branch point for the elaboration of the various flavonoid and isoflavonoid secondary metabolites found in the plant kingdom. Various *in vivo* labeling experiments prior to 1984 (reviewed by Dixon *et al.*[174]) had demonstrated that chalcone/flavanone was incorporated into isoflavonoids. For example, Grisebach and Brandner[175] showed that 2′,4,4′-trihydroxychalcone 4-*O*-glucoside (**63**) was converted to daidzein (**4**) in chickpea, although at very low levels, and (2*S*)-naringenin (**61**) was stereoselectively converted to biochanin A (**33**), a finding that was interpreted as indicating that the flavanone rather than its nonoptically active isomeric chalcone was the substrate for the proposed enzyme-catalyzed B-ring aryl migration. Such a reaction was assumed to be oxidative, leading to the isoflavone as the first isoflavonoid product. At the same time, putative mechanisms for such a reaction were proposed, based either on theoretical considerations or on results of direct chemical oxidations of flavonoid compounds. Such mechanisms included epoxidation and/or the formation of a spirodienone intermediate with aryl migration associated with *O*-methylation at the 4′-position (Scheme 7).

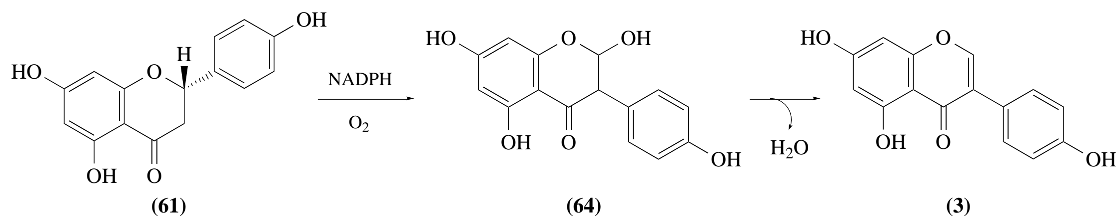


In 1984, Hagmann and Grisebach[176] provided the first evidence for the enzymatic conversion of flavanone to isoflavone (the "isoflavone synthase" (IFS) reaction) in a cell-free system. They demonstrated that microsomes from elicitor-treated soybean cell suspension cultures could catalyze the conversion of (**61**) to genistein (**3**) or of (2-*S*)-liquiritigenin (**60**) to (**4**) in the presence of NADPH. The crude microsomal enzyme preparation, which was stable at −70 °C but had a half-life of only 10 min at room temperature, was absolutely dependent on NADPH and molecular oxygen. It was



Scheme 7

subsequently shown[177] that the reaction proceeded in two steps. Naringenin was converted in a cytochrome P450-catalyzed reaction requiring NADPH and O_2 to the corresponding 2-hydroxyisoflavanone (**64**). This relatively unstable compound then underwent dehydration to yield (**3**) (Scheme 8). The dehydration reaction appeared to be catalyzed by an activity present predominantly in the cytoplasmic supernatant, although it was not possible to remove all this activity from the microsomes. Compound (**64**) can spontaneously convert to (**3**), for example in MeOH at room temperature. Kinetic analysis indicated that (**64**) is formed prior to (**3**), consistent with its being an intermediate.



Scheme 8

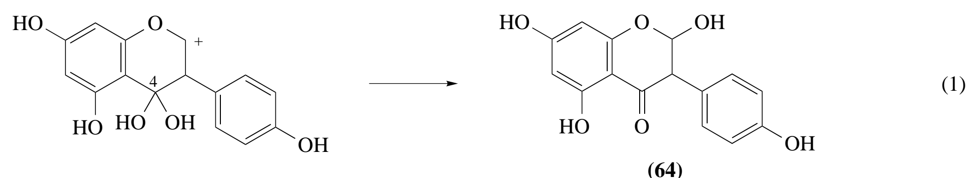
Involvement of cytochrome P450 in the 2-hydroxyisoflavanone synthase reaction was confirmed by inhibition by CO, replacing O_2 with N_2, and a range of known P450 inhibitors of which ancymidol was the most effective. The enzyme comigrated with the endoplasmic reticulum markers cinnamate 4-hydroxylase and cytochrome b5 reductase on Percoll gradients. The properties of the crude microsomal IFS are shown in Table 4. The enzyme is stereoselective, and (2*R*)-naringenin (**62**) is not a substrate.

Table 4 Properties of isoflavone synthase activity in microsomes from elicited cell suspensions of *Glycine max* and *Pueraria lobata*.

Property	*G. max*	*P. lobata*
Specific activity:		
Unelicited	not reported	76 nkat kg^{-1}
Elicited	302 nkat kg^{-1}	1164 nkat kg^{-1}
Optimum pH	8.0–8.6	not reported
K_m:		
naringenin (**61**)	8.7 μmol L^{-1}	20 μmol L^{-1}
liquiritigenin (**60**)	not reported	6.9 μmol L^{-1}
NADPH	39 μmol L^{-1}	not reported
$t_{1/2}$ at 4°C	200 min	60 h

Source: Kochs and Grisebach[177] and Hakamatsuka *et al.*[178]

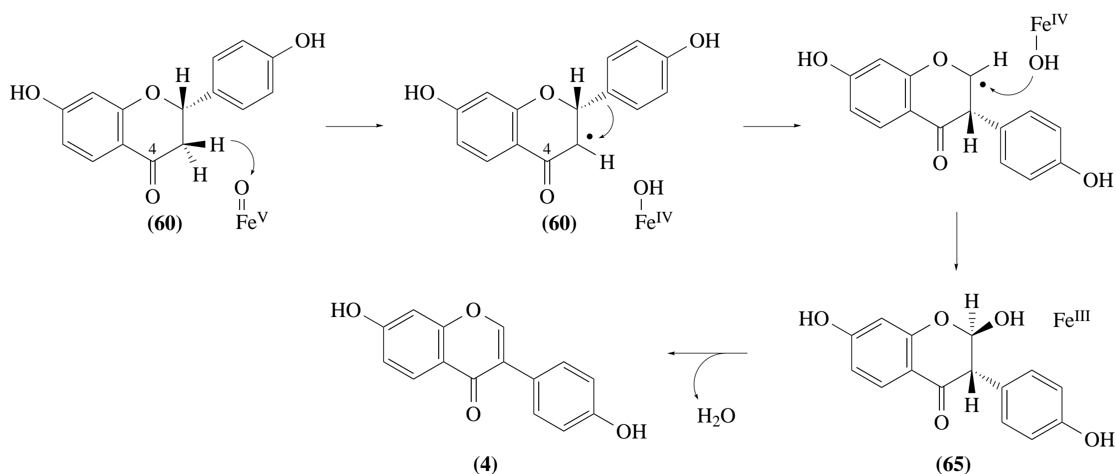
The pioneering work of Grisebach's group did not unequivocally address the origin of the 2-hydroxyl group (i.e., from molecular oxygen or water). Indeed, their model for the reaction involved the initial formation of a diol at position C-4 with subsequent addition of a hydroxyl to the carbocation formed at position 2 (Equation (1)). The origin of the 2-hydroxyl group was determined from studies on the isoflavone synthase present in microsomes from elicited cell cultures of *Pueraria lobata*, some properties of which are summarized in Table 4. Carefully washed microsomes produced predominantly (**65**) from (**60**), whereas (**4**) was the only product in cruder microsome preparations. As with the soybean system, a soluble enzyme was shown to catalyze the dehydration of (**65**) to (**4**).[179] ^{18}O from $^{18}O_2$ was incorporated into the 2-hydroxyl group, resulting in a 2-hydroxyisoflavanone with the molecular ion shifted by two mass units, whereas there was no corresponding shift in the molecular ion of (**4**), consistent with the subsequent dehydration reaction. Furthermore, use of [4-^{18}O]-(**60**) as substrate demonstrated that there was no exchange of the carbonyl oxygen, a finding which disproves the earlier suggestion of the formation of a diol at position C-4. The currently accepted model for the reaction pathway of "isoflavone synthase" (Scheme 9)[180] therefore involves P450-catalyzed hydroxylation coupled to aryl migration, a reaction with mechanistic similarities to the well-described proton migration mechanism of some P450 reactions. Similarities between the mechanism of IFS and other reactions such as ring condensation of *ent*-7-hydroxykaurenoic acid to GA_{12} aldehyde in gibberellin biosynthesis, formation of the furan ring in furanocoumarin synthesis, and sterol demethylation have been discussed.[180]



(1)

Dual-labeling experiments with [^{14}C]chalcone and [^{3}H]flavanone confirmed that the flavanone and not the chalcone was the substrate for the *Pueraria* IFS.[178] This confirms the role of chalcone isomerase as a key enzyme of isoflavonoid synthesis. The *Pueraria* IFS prefers the 5-deoxyflavanone (**60**) to (**61**), and this is reflected by the chalcone isomerase from cell cultures of this species being active against (**36**) but not (**42**).[178]

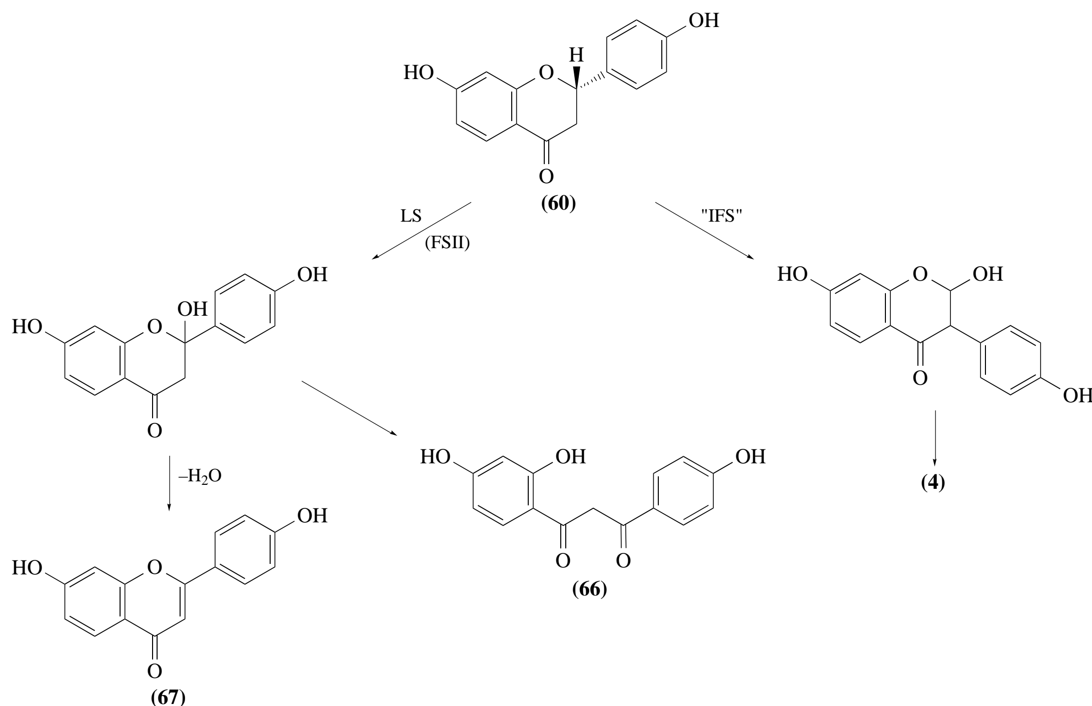
There have been no reports on the purification to homogeneity, or the molecular cloning, of either of the two enzymes of the IFS complex. The flavanone 2-hydroxylase cytochrome P450 from *Pueraria* has been solubilized with Triton X-100 and partially purified by DEAE-Sepharose chromatography; the enzymatic reaction could be reconstituted by addition of NADPH cytochrome P450 reductase that separated from the hydroxylase on the ion-exchange column.[177] The 2-hydroxy-



Scheme 9

isoflavanone dehydratase has been purified from elicitor-treated *P. lobata* cells, and is a soluble monomeric enzyme of subunit M_r 38 000.[101] It is not clear whether this enzyme physically associates with the P450 hydroxylase catalyzing the aryl migration.

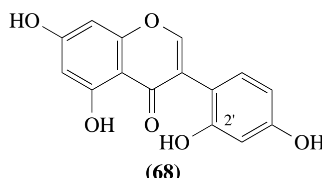
Flavanone is a potential substrate for more than one type of hydroxylation reaction at the 2-position. Thus, elicitor-treated cell cultures of alfalfa and *Glycyrrhiza echinata* have been shown to accumulate the dibenzoylmethane licodione (**66**).[181,182] Licodione synthase is, by classical criteria, a cytochrome P450, the activity of which is induced by yeast elicitor in *Glycyrrhiza* cells.[182] The reaction it catalyzes involves 2-hydroxylation of flavanone followed by hemiacetal opening, and may have mechanistic similarities to the flavone synthase II enzyme previously characterized from soybean.[183] A comparison of the reactions catalyzed by IFS, licodione synthase, and flavone synthase II (leading to the formation of 7,4′-dihydroxyflavone (**67**)) is shown in Scheme 10.



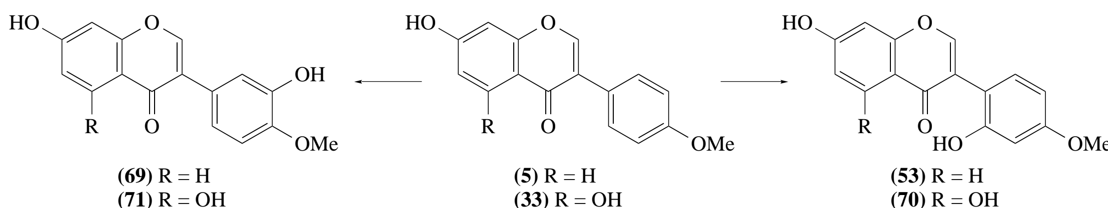
Scheme 10

1.28.3.7 2′- and 3′-Hydroxylation of Isoflavones

In addition to the formation of 2-hydroxyflavanone (**64**) and genistein (**3**), soybean microsomes incubated with naringenin (**61**) also produced small amounts of 2′-hydroxygenistein (**68**), indicating the presence of an isoflavone 2′-hydroxylase activity.[177] 2′-Hydroxylation is a prerequisite for subsequent reduction and ring closure in the formation of pterocarpans, and elicitor-induced increases in microsomal isoflavone 2′-hydroxylase activities have been described in cell cultures of alfalfa[184] and chickpea,[185–187] associated with phytoalexin accumulation.



Microsomes isolated from yeast elicitor-treated chickpea cell cultures catalyzed the formation of 2′-hydroxyformononetin (**53**) and calycosin (3′-hydroxyformononetin) (**69**) from formononetin (**5**) (K_m values 3.3 and 11.0 μmol L^{-1}, respectively), and 2′-hydroxybiochanin A (**70**) and pratensin (3′-hydroxybiochanin A) (**71**) from biochanin A (**33**) (K_m values 13.0 and 12.5 μmol L^{-1}, respectively) (Scheme 11). No 2′,3′-dihydroxylated products were formed. No activity was observed with daidzein (**4**) or (**3**) as substrates, suggesting that, at least in chickpea, 4′-*O*-methylation is a prerequisite for 2′-hydroxylation. Furthermore, no activity was observed with formononetin-7-ethyl ether (**72**), (**53**), vestitione (**54**), or medicarpin (**6**). The 2′-hydroxylation reactions were shown to be catalyzed by a cytochrome P450 enzyme system on the basis of inhibition with cytochrome *c*, juglone, or flushing with nitrogen.[185,187]



Scheme 11

Several pieces of indirect evidence point to there being more than one enzyme involved in the 2′- and 3′-hydroxylation of chickpea isoflavones. Thus, the optimum pH for 2′-hydroxylation is 7.4, whereas that for 3′-hydroxylation is 8.0.[187] The cytochrome P450 inhibitors BAS 110 and BAS 111 have differential effects on 2′- and 3′-hydroxylase activities. Finally, the induction kinetics of the 2′- and 3′-hydroxylase activities are different in both cell cultures and roots.[186,187] It is still not clear whether one or two enzymes catalyze the 2′-hydroxylation of (**5**) and (**33**).

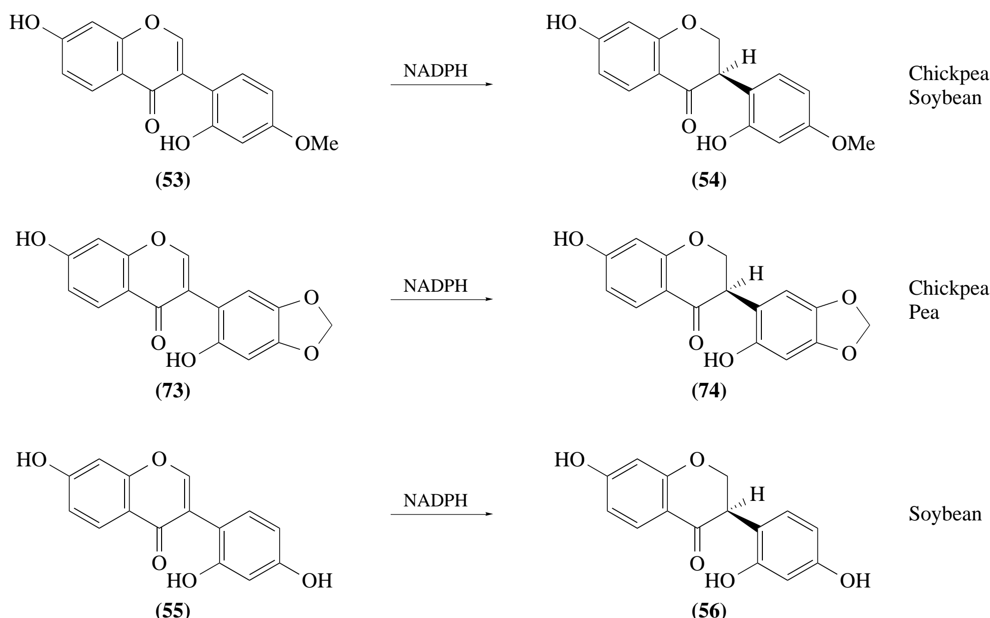
It is interesting to note that, unlike the situation in chickpea, 2′-hydroxylase activity in soybean, a species that does not appear to accumulate 4′-methoxyisoflavonoids, does not require a 4′-methoxylated isoflavone as substrate.[177] Compound (**5**) is a substrate for 2′-hydroxylation in alfalfa, but no 3′-hydroxylase activity appears to be present in microsomes from elicited alfalfa cells.[184] The 3′-hydroxylation reaction observed in chickpea microsomes is presumably involved in the synthesis of (−)-maackiain (**59**), which has a 3′,4′-methylenedioxy substituent. It is probable that 3′-hydroxylation and formation of the methylenedioxy bridge occur prior to 2′-hydroxylation during the biosynthesis of (**59**) in chickpea[185] (see Section 1.28.3.10.2).

Comparative studies of the activities of a range of enzymes involved in the formation of medicarpin in elicited chickpea cell cultures from lines resistant or susceptible to the fungal pathogen *Ascochyta rabiei* have indicated that the increased production of (**6**) in the resistant line is most likely determined by its high activity of formononetin 2′-hydroxylase.[186]

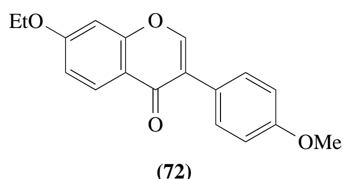
1.28.3.8 Conversion of Isoflavone to Isoflavanone

Reduction at the C-2 atom of the heterocyclic ring of isoflavones leads to the formation of isoflavanones, which are obligatory intermediates in the formation of pterocarpans.[130,188] *In vivo*

labeling and early enzymological studies confirmed that 2′-hydroxylation is a prerequisite for enzymatic reduction of isoflavone to isoflavanone. A crude enzyme preparation from yeast elicitor-treated chickpea cell suspension cultures was shown to catalyze the NADPH-dependent reduction of 2′-hydroxyformononetin (**53**) to vestitione (**54**); the only other substrate was the methylenedioxy-substituted isoflavone 2′-hydroxypseudobaptigenin (**73**), which is converted to (−)-sophorol (**74**) (Scheme 12).[189] This activity was rapidly and strongly induced in response to elicitors in a chickpea cell line that accumulated medicarpin, but was only weakly induced in a line that produced little phytoalexin and was susceptible to the fungus *Ascochyta rabiei*.[109,189]



Scheme 12



The chickpea isoflavone reductase (IFR) has been purified to homogeneity and a cDNA clone obtained.[190] The highly purified enzyme is a mononer of M_r 36 000, and has K_m values of 6, 6, and 20 μmol L^{-1} for (**53**), (**73**), and NADPH, respectively.[190] Substrate specificity studies confirmed absolute requirements for the 2′-hydroxyl group and either a 4′-methoxy or 4′,5′-methylenedioxy substitution on the B-ring. This contrasts with the enzyme from soybean, a species that does not produce 4′-*O*-methylated isoflavonoids. Soybean IFR has been purified to homogeneity from elicitor-treated cell cultures. Although having an absolute requirement for the 2′-hydroxyl substitution pattern, it can convert 2′-hydroxydaidzein (**55**) to 2′-hydroxydihydrodaidzein (**56**) (K_m 50 μmol L^{-1}) and also (**53**) to (**54**) (K_m 60 μmol L^{-1}.)[191]

Extracts from $CuCl_2$-elicited pea seedlings, which accumulate the methylenedioxy-substituted pterocarpans (+)-maackiain (**52**) and (+)-pisatin (**21**), can catalyze the reduction of (**73**) to (**74**). This activity is strongly induced on elicitation in parallel with that of a 6a-hydroxymaackiain-3-*O*-methyltransferase.[192] It would appear, from the substrate specificities of the IFRs from chickpea, soybean, and pea, that reduction of isoflavone to isoflavanone generally occurs after B-ring methylation in species that make B-ring methoxy isoflavanone-derived compounds.

Antibodies to the soybean IFR protein recognize IFR and two additional bands of slightly higher M_r on Western blots of crude soybean protein extracts.[191] Likewise, antibodies against a pea IFR recognize multiple bands, one constitutive and two induced, on Western blots of elicited alfalfa cell cultures,[193] suggesting that plants may contain multiple IFR-like proteins (see Section 1.28.6).

Reduction of the C-2 atom of isoflavones generates a chiral center. The CD spectrum of the product formed from (**55**) by the purified soybean IFR confirmed the 3*R* stereochemistry of the isoflavanone.[191] Studies with stereospecifically tritiated NADPH suggested *trans*-addition from H_A of NADPH to the C-2 atom of (**53**) by the chickpea IFR.[194] This has been confirmed using recombinant alfalfa IFR expressed in *E. coli* to catalyze the formation of (3*R*)-vestitone (**54**) from (**53**).[193] However, the CD spectrum of (**74**) produced from (**73**) by recombinant pea IFR expressed in *E. coli* also revealed the 3*R* stereochemistry,[195] which was unexpected because the final pterocarpan products in pea, (+)-maackiain (**52**) and (+)-pisatin (**21**), have the opposite stereochemistry at their two chiral centers (6a and 11a) compared with the (−)-medicarpin (**6**), (−)-maackiain (**59**), or (−)-3,9-dihydroxypterocarpan (**57**) produced in alfalfa, chickpea, or soybean. This problem is discussed further in Section 1.28.3.9.

Amino acid sequence data indicate a high degree of similarity between the IFRs cloned from chickpea,[190] alfalfa,[193] and pea.[195] Alfalfa IFR is 92% identical with pea IFR at the overall amino acid level, and shares 62% identity in its *N*-terminal region to *Antirrhinum* dihydroflavanol reductase. IFR is encoded by a single gene in pea and alfalfa, and its transcripts are highly induced in response to elicitors.[193,195] This induction has been shown, by nuclear transcript run on analyses, to be the result of increased *de novo* transcription rather than effects on RNA stability.[159,196]

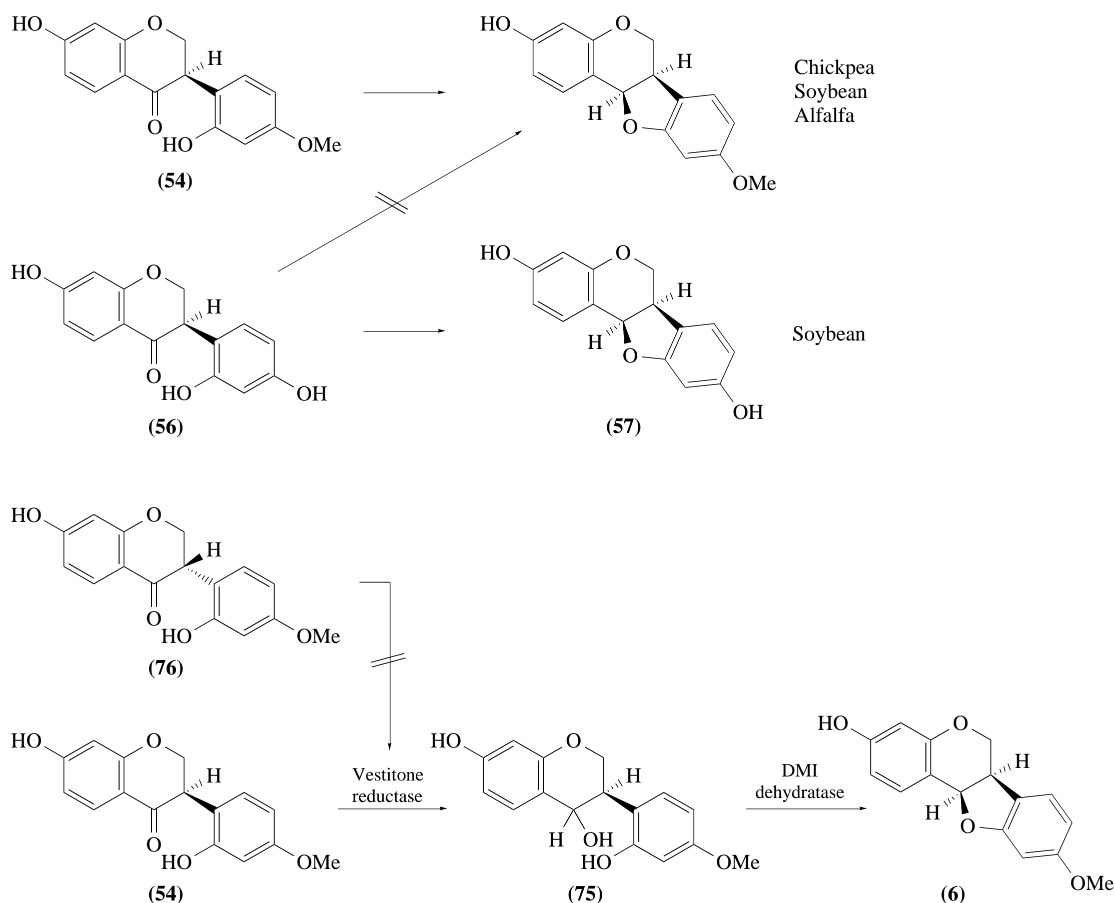
Northern blot analysis has indicated that IFR transcripts in alfalfa are most abundant in roots and nodules, consistent with the constitutive accumulation of isoflavanoid malonyl glycosides in these organs.[193] This same pattern of expression is observed for the alfalfa IFR promoter driving expression of the β-glucuronidase (GUS) reporter gene in transgenic alfalfa, although unexpected ectopic expression in a range of tissues was observed following transformation of tobacco with IFR–GUS fusions.[196]

1.28.3.9 Isoflavanone Reductase and the Synthesis of Pterocarpans

Pterocarpans contain a fused furan ring structure that arises from ring closure between the C-4-carbonyl and C-2′-positions of 2′-hydroxyisoflavanones. The mechanism of this reaction has been elucidated following the characterization of the enzymatic system involved in this late stage of isoflavonoid phytoalexin synthesis.

The first report of the *in vitro* formation of pterocarpans was the demonstration of the NADPH-dependent conversion of vestitone (**54**) to (6a*R*, 11a*R*)-medicarpin (**6**) catalyzed by a soluble enzyme extract from yeast elicitor-treated chickpea cell suspensions.[197] The greater incorporation into (**6**) of optically active (−)-(**54**) than the racemic mixture indicated a stereochemical preference for the 3*R* optical isomer of (**54**) by the enzyme system. The enzyme was partially purified through ammonium sulfate precipitation and ion-exchange chromatography, and appeared to elute as a single activity, optimum pH 6.0, with K_m values for (**54**) and NADPH of 17 μmol L^{-1} and 40 μmol L^{-1} respectively.[197] Essentially similar observations were made in the case of the enzyme system from elicited soybean cell suspensions,[198] which was purified only 7.3-fold by a five-step procedure including ion-exchange chromatography, blue Sepharose (to which the enzyme did not bind), and gel filtration (revealing an M_r of 29 000). This enzyme converted (3*R*)-2′-hydroxydihydrodaidzein (**56**) to (6a*R*, 11a*R*)-3,9-dihydroxypterocarpan (**57**), with an optimum pH of 6.0 and K_m values for the isoflavanone and NADPH of 75 μmol L^{-1} and 45 μmol L^{-1}, respectively. Compound (**54**) was converted to (**6**) at approximately half of the rate for the conversion of (**56**) to its corresponding pterocarpan. In contrast, the chickpea enzyme has an absolute requirement for the presence of the 4′-methoxy group of (**54**), and (**56**) is therefore not a substrate (Scheme 13).

Both of the above reports suggested that pterocarpan formation was catalyzed by a single enzyme, which was termed "pterocarpan synthase," and this activity, with 2′-hydroxyisoflavanone as substrate, is induced by elicitor treatment when measured in crude extracts from chickpea, soybean, and alfalfa cell suspension cultures.[193,197,198] Bless and Barz[197] indicated the possibility that the reaction might proceed through an isoflavan-4-ol intermediate (**75**) (Scheme 13). Confirmation of this came from studies on the "pterocarpan synthase" from elicited alfalfa cell suspension cultures.[199] Attempted purification on red agarose indicated that an enzyme activity consuming (**54**) was bound to the column, but, on elution, this activity did not form medicarpin (**6**). Rather, an intermediate was formed which could be converted to (**6**) by a second enzyme present in the flow-through fraction from the red agarose affinity column. The intermediate compound was shown to be 7,2′-dihydroxy-4′-methoxyisoflavanol (DMI) (**75**) when (**54**) was used as substrate. The "pterocarpan synthase" reaction was therefore catalyzed by two enzymes, a reductase (in the case



Scheme 13

of alfalfa a vestitone reductase) that converts the 2′-hydroxyisoflavanone to its corresponding isoflavanol, and a dehydratase that catalyzes the final ring closure. Alfalfa vestitone reductase and DMI dehydratase were both extensively purified.[199] The reductase is a monomeric enzyme of subunit M_r 38 000, optimum pH 6.0, with a K_m value for (**54**) of 40 μmol L^{-1}. The activity is inhibited by concentrations of (**54**) above 50 μmol L^{-1}. The enzyme was specific for (3*R*)-(**54**), and (3*S*)-vestitone (**76**) did not inhibit the enzyme. DMI dehydratase has a native M_r of 38 000, optimum pH 6.0, and a K_m value for (**75**) of 5 μmol L^{-1}. It produces only (6a*R*, 11a*R*)-medicarpin (**6**) from (3*R*)-vestitone (**54**).[200] It appears to be a very hydrophobic enzyme, and can form a physical association with vestitone reductase *in vitro* at low salt concentrations.[200] The dehydratase is coinduced with the reductase in elicitor-treated alfalfa cells.[200]

It is very likely that the "pterocarpan synthases" from chickpea and soybean also consist of a separate reductase and dehydratase. This would explain why multi-step purification protocols only gave very restricted purifications from these sources,[197,198] the reductase and dehydratase will partially copurify on size-exclusion chromatography, and a small amount of dehydratase may then fractionate with the reductase on other matrices due to protein–protein interactions. It will be interesting to determine whether the reductase and dehydratase are physically associated *in vivo*.

1.28.3.10 Substitution of the Isoflavonoid Nucleus

1.28.3.10.1 O-Methylation

The isoflavonoid phytoalexins of several species, including alfalfa and chickpea, are methylated at the 4′-position of the B-ring (Figure 1). However, in spite of many metabolic and enzymatic studies, the nature of the enzymatic step resulting in this methylation is still unclear.

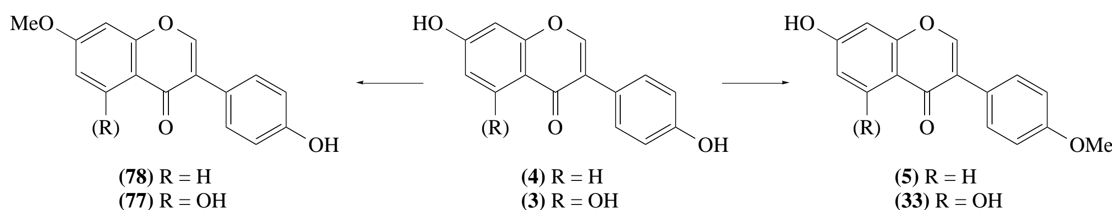
Radiolabeled precursor feeding experiments with elicited alfalfa seedlings indicated that, although 2′,4,4′-trihydroxychalcone (**36**) and formononetin (**5**) were good precursors of medicarpin (**6**), daidzein (**4**) (the presumed substrate of the 4′-*O*-methyltransferase) was not incorporated.[130] These results were originally interpreted as indicating a requirement for methylation of the B-ring during the aryl migration reaction catalyzed by isoflavone synthase[130] (see Scheme 7). This now seems unlikely in view of the demonstration that the aryl migration catalyzed by the 2-hydroxyisoflavanone synthase described in Section 1.28.3.6 can occur in the absence of methylation in species in which the 4′-hydroxyl group is either free (e.g., in soybean)[177] or methylated (e.g., in alfalfa).[184] Our knowledge of the substrate specificities of the enzymes preceding and following (**4**) in the isoflavonoid pathway points to (**4**) as the substrate for 4′-*O*-methylation. Furthermore, a mutant of subterranean clover (*Trifolium subterraneum*), which produced greatly reduced levels of (**5**) and biochanin A (**33**), accumulated high levels of (**4**), suggesting that (**4**) is the immediate precursor of (**5**)[201] (Table 5). Normally, free (**4**) is present at very low levels in subterranean clover (and in many other legumes), although genistein (**3**) can accumulate to appreciable levels (Table 5).

Table 5 Levels of isoflavonoids in two varieties and one mutant line (A258) of subterranean clover.

Isoflavone	*Clare*	*Geraldton*	*Geraldton A*258
Genistein (**3**)	242	14.6	60.5
Biochanin A (**33**)	4.1	23.4	0.4
Pratensin (**71**)	0	1.6	trace
Daidzein (**4**)	0	0.2	76.5
Formononetin (**5**)	4.0	42.7	4.0

Source: Wong and Francis[201,202].

The contradiction between the labeling studies described above (which should be reevaluated in a cell culture system which is more optimal for precursor uptake) and the enzymological and genetic studies pointing to (**4**) as a substrate for 4′-*O*-methylation is compounded by attempts to demonstrate the enzymatic basis for the origin of the 4′-methoxy group of isoflavones. It would be expected that a simple isoflavone 4′-*O*-methyltransferase reaction would be involved in the conversion of (**4**) to (**5**), or of (**3**) to (**33**) (Scheme 14). However, in a study of isoflavone 4′-*O*-methylation in chickpea cell cultures, an isoflavone 7-*O*-methyltransferase activity, which methylated the A-ring of (**3**) to yield prunetin (5,4′-dihydroxy-7-methoxyisoflavone) (**77**) was described[176] (Scheme 17). This enzyme activity had been initially described as a 4′-*O*-methyltransferase occurring as a dimer of M_r 110 000, with an optimum pH of 9.0 and a K_m for (**4**) of 80 μmol L^{-1}.[203]



Scheme 14

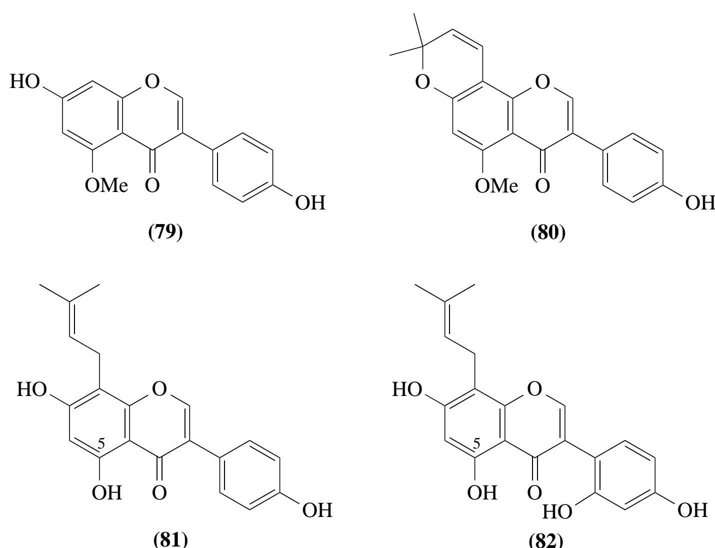
Fungal infection of jackbean (*Canavalia ensiformis*) callus led to a 3–4-fold increase in the extractable activities of enzymes that could methylate (**4**) and (**3**), and it was reported that the products cochromatographed with the 4′-methoxy derivatives.[204] However, treatment of alfalfa cell suspension cells with yeast elicitor results in a massive induction of isoflavone 7-*O*-methyltransferase activity,[205] which methylates the A-ring of (**4**) to produce isoformononetin (4′-hydroxy-7-methoxy-isoflavone) (**78**)[205] (Scheme 14), a rare naturally occurring compound which is unlikely to be involved in the formation of medicarpin (**6**). The enzyme, which is monomeric, was purified by SDS-PAGE to a single band of M_r 41 000 that could be photoaffinity labeled with [^{3}H]-*S*-adenosyl-L-methionine,[205] although the preparation was contaminated with high levels of caffeic acid 3-*O*-methyltransferase (COMT) activity. Partially purified alfalfa isoflavone 7-OMT had an optimum pH of 8.5, a K_m value of 20 μmol L^{-1} for (**4**), and exhibited a very low level of 4′-*O*-methyltransferase activity resulting in the formation of (**5**).[205] It has not proven possible to purify this 4′-OMT activity further.[206] The extremely low level of daidzein 4′-*O*-methyltransferase activity in elicited alfalfa

cultures contrasts with the strongly increased extractable activity of the isoflavone 7-*O*-methyltransferase in parallel with all the other known enzymes in the pathway leading to (**6**).[205,207]

The author's research group has developed a substrate-based affinity chromatographic system to purify the 41 kDa isoflavone 7-OMT to homogeneity.[206] Four internal peptide sequences were obtained from the purified protein, one of which had high (72%) sequence identity with a region of a catechol-*O*-methyltransferase from barley. All four internal peptides had about 55% amino acid sequence identity with four regions of 6a-hydroxymaackiain 3-*O*-methyltransferase from *Pisum sativum* (see below), but had no sequence identity with the alfalfa COMT or chalcone 2′-*O*-methyltransferase (ChalOMT) genes previously cloned. The purified isoflavone *O*-methyltransferase had substrate specificity toward isoflavones with a free 7-hydroxyl group, and could also methylate the 5-hydroxyl group of (**3**). It was inactive against (**5**).

It is proposed that the enzyme with isoflavone 7-OMT activity *in vitro* may methylate the 4′-position *in vivo*. The unexpected precursor feeding results in alfalfa can be explained if the OMT is in a "metabolic compartment" or "channel," and its association with the enzymes producing its substrate or removing its product could account for the different product specificities observed *in vivo* and *in vitro*. The isoflavone synthase and 2′-hydroxylase are both microsomal cytochrome P450s, with which the 4′-OMT could be physically associated. Thus, only (**4**) formed *in situ* by microsomal isoflavone synthase, but not exogenously supplied (**4**), might act as substrate for the OMT (Figure 1). This hypothesis can be tested by molecular genetic strategies. To this end, full-length cDNA clones encoding the isoflavone OMT have been obtained. These can be transformed into plant cells that normally produce isoflavonoids with a free 4′-hydroxyl group, such as green bean or soybean, and the effects on metabolites determined. At the same time, testing is continuing to determine whether the isoflavone OMT can be used to identify other interacting proteins, using the yeast two-hybrid system for cloning genes based on physical interactions between their products.[208]

The 5-hydroxyl group of isoflavones is energetically the most difficult to methylate owing to its chelation to the carbonyl oxygen of the heterocyclic ring. However, yellow lupin roots accumulate a range of 5-methoxyisoflavones (e.g., 5-*O*-methylgenistein (**79**) and 5-*O*-methylderrone (**80**)) based on (**3**) and its 8-prenyl derivative (**81**). Khouri *et al.*[209] have reported the 810-fold purification of an isoflavone 5-*O*-methyltransferase from this source. The enzyme is a monomer of subunit M_r 55 000, with a pH optimum of 7.0 and a K_m value for (**3**) of 1 μmol L^{-1}. Its substrates, in order of decreasing activity, are 8-prenyl-2′-hydroxygenistein (**82**), 2′-hydroxygenistein (**68**), (**3**), and (**81**). This suggests that methylation of the 5-position can occur at several stages during the biosynthesis of the lupin isoflavonoids.



The final step in the biosynthesis of the pea phytoalexin (+)-pisatin (**21**) is the *O*-methylation of the 3-position of the pterocarpan (+)-6a-hydroxymaackiain (**43**) (Equation (2)). The 3-position of a pterocarpan is equivalent to the 7-position of the isoflavone nucleus. Preisig *et al.*[210] have purified an enzyme from $CuCl_2$-treated pea seedlings that catalyzes this methylation reaction. The monomeric enzyme has a subunit M_r of 43 000, exists as two isoforms of p*I* 5.2 and 4.9, and has an optimum

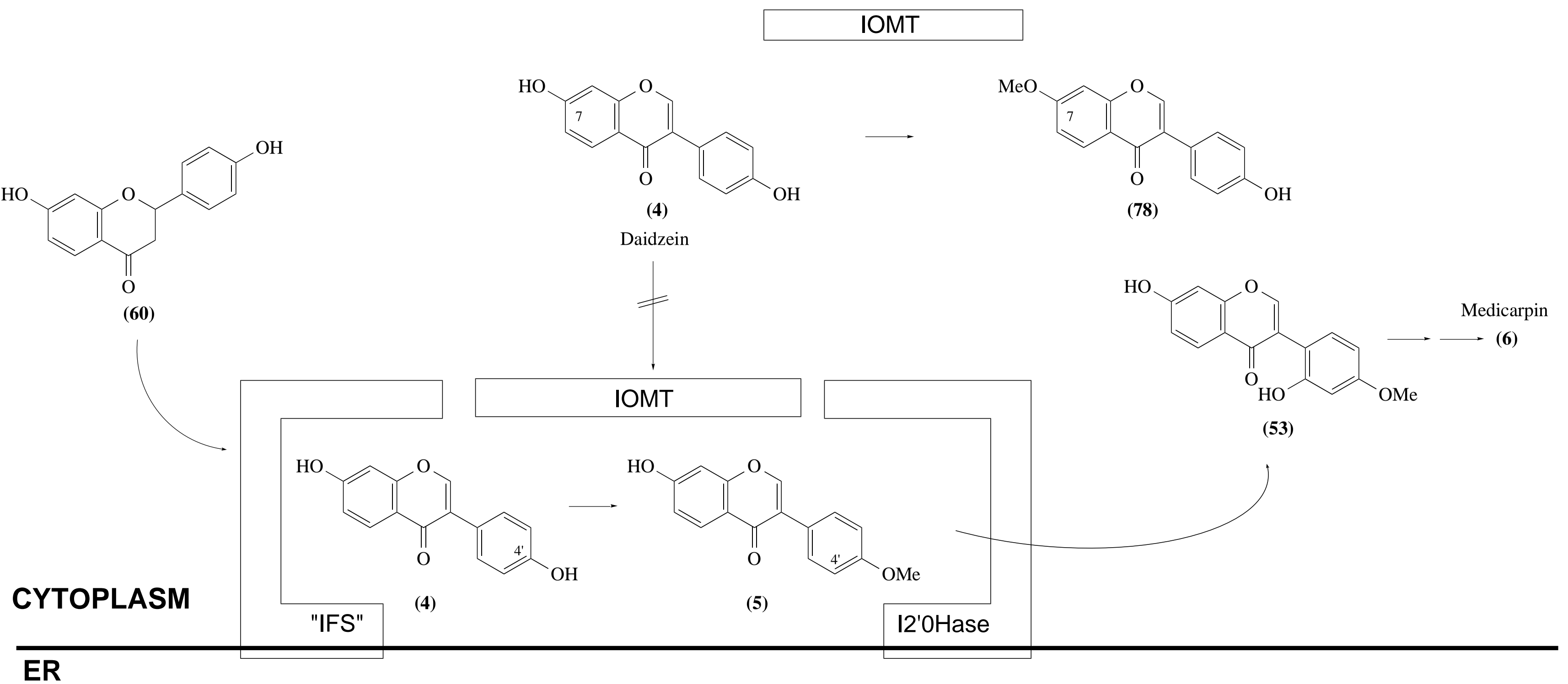
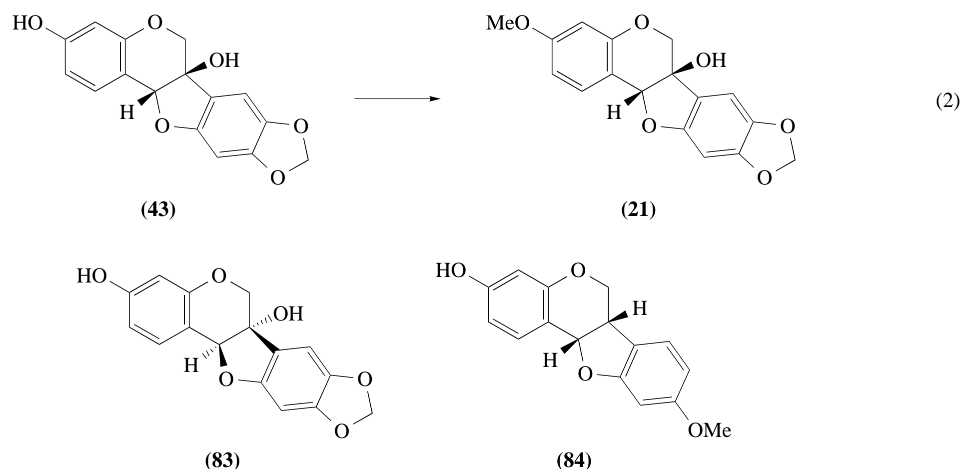


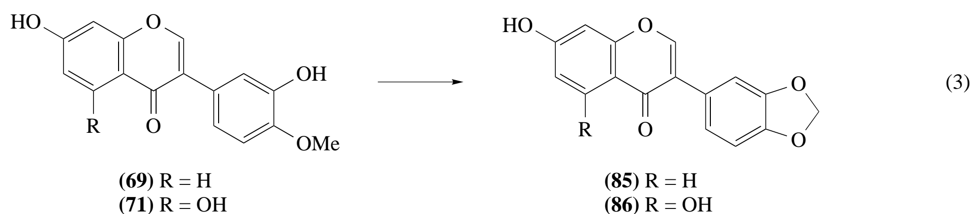
Figure 1 Potential metabolic channeling involving isoflavone *O*-methyltransferase. Association of the enzyme with endoplasmic reticulum (ER)-associated cytochrome P450 systems that catalyze isoflavone formation and 2′-hydroxylation is proposed to alter the product specificity of the enzyme, resulting in synthesis of the B-ring methylated isoflavone formononetin (**5**) rather than the A-ring methylated isoformononetin (**78**) which is the major product of the enzyme *in vitro*.

pH of 7.9 and a K_m value for (**43**) of 2.3 μmol L^{-1}. The enzyme has highest activity against (**43**), and low activity against (−)-6a-hydroxymaackiain (**83**), (+)-maackiain (**52**), and (+)-medicarpin (**84**). Antibodies were raised against the purified enzyme and used to demonstrate induction of enzyme protein and translatable mRNA activity in response to elicitation.[211] A cDNA encoding this OMT has been cloned;[212] it shows 51% amino acid sequence identity with the isoflavone 7-OMT from alfalfa.[213]



1.28.3.10.2 *Formation of methylenedioxy rings*

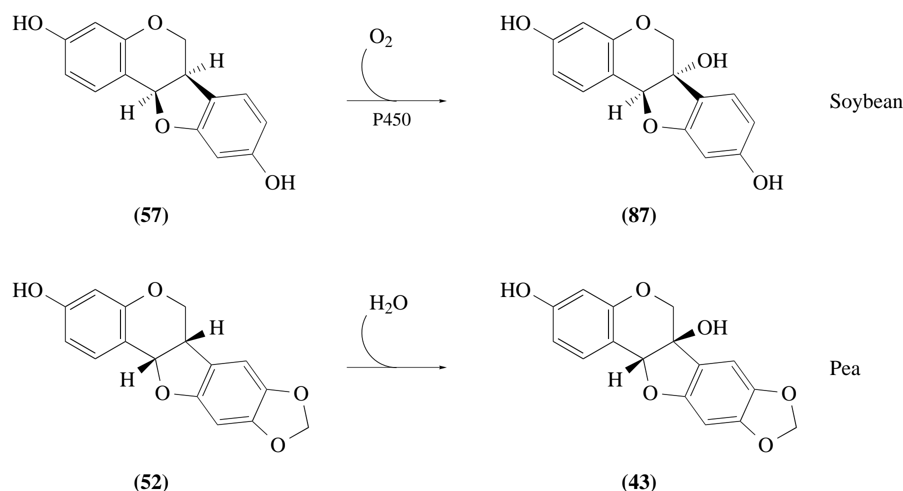
Methylenedioxy bridge functions occur in a number of isoflavonoids, the best known of which are the pterocarpan phytoalexins maackiain (**52**) and pisatin (**21**). Studies with elicitor-treated chickpea cell suspensions have indicated that maackiain synthesis proceeds via cytochrome P450-mediated 3′-hydroxylation of formononetin (**5**) to yield calycosin (**69**) (see above), followed by methylenedioxy bridge formation to yield pseudobaptigenin (**85**) (Equation (3)). A parallel series of reactions occur with the 5-hydroxylated isoflavone pratensin (**71**), although further metabolism of the methylenedioxy-substituted product leads to a substituted isoflavanone rather than to a pterocarpan. Chickpea microsomes convert (**69**) and (**71**) to (**85**) and 5′-hydroxypseudobaptigenin (**86**), respectively, in a reaction requiring oxygen and NADPH that shows all the classical characteristics of a cytochrome P450-catalyzed reaction.[214] This enzyme system has apparent K_m values for (**69**) and NADPH of 2 μmol L^{-1} and 70 mol L^{-1}, respectively, and is strongly induced, from a zero background level, in response to elicitor.



1.28.3.10.3 *6a-Hydroxylation of Pterocarpans*

The 6a position is hydroxylated in several pterocarpan phytoalexins such as pisatin (**21**) from peas and the glyceollins (**18–20**) from soybean. Fungal degradation of pterocarpans can also involve hydroxylation of the 6a position (see Section 1.28.4.2). The mechanism of 6a-hydroxylation of the glyceollin precursor 3,9-dihydroxypterocarpan (**57**) in soybean has been clearly established (Scheme 15). The cytochrome P450 enzyme catalyzing the formation of 3,6a,9-trihydroxypterocarpan (**87**) was solubilized from soybean microsomal membranes utilizing 1% Chaps detergent,[215] although this detergent strongly inhibits the hydroxylase at concentrations below the critical micellar concentration. In spite of low recovery, the enzyme was purified 765-fold to yield a major component of subunit M_r 55 000, the activity of which could be reconstituted with purified soybean NADPH:

cytochrome P450 reductase in the presence of added lipid, with dilauroylphosphatidylcholine being the most effective. The pH optimum of the enzyme was 7.0. The enzyme could be resolved from the more abundant cinnamate 4-hydroxylase cytochrome P450, and this was in fact the first direct demonstration of the presence of distinct molecular species of P450s with different catalytic activities in plants. Soybean pterocarpan 6a-hydroxylase activity was not detectable in unelicited cells.



Scheme 15

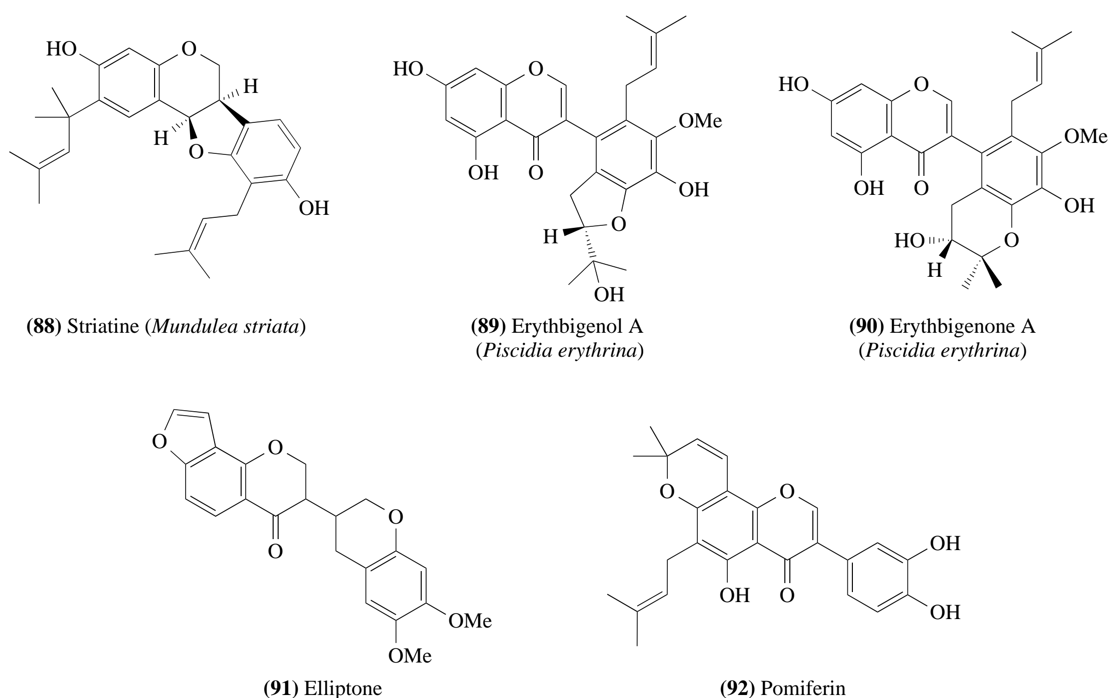
In soybean, the pterocarpan precursor for 6a-hydroxylation is of the (−)-6a*R*,11a*R* stereochemistry, whereas in peas the maackiain (**52**) that is hydroxylated in the penultimate step of pisatin (**21**) biosynthesis is of the opposite stereochemistry. ^{18}O-labeling experiments with $CuCl_2$-treated pea seedlings led to the surprising conclusion that the 6a-hydroxyl group of (**52**) destined for biosynthesis of (**21**) is derived from water rather than from molecular oxygen[216] (Scheme 15). This contrasts with the fact that fungal degradation of maackiain proceeds via 6a-hydroxylation utilizing molecular oxygen although, in this case, the maackiain that the fungus degrades in this manner is the (−)-stereoisomer.[216]

It is not clear at present whether these different mechanisms for 6a-hydroxylation of (6a*R*, 11a*R*)- and (6a*S*, 11a*S*)-pterocarpans are associated with an as yet unidentified pathway for the biosynthesis of the (+)-pterocarpans. The finding that the pea IFR produces the 3*R* steroisomer of sophorol (**74**)[195] raises questions about the mechanism of (+)-pterocarpan formation. The problem is how (**74**) is converted to (**21**). (+)-6a-Hydroxymaackiain (**52**) is clearly the immediate substrate for the methyltransferase that is the final step in pisatin biosynthesis.[210] Production of the (+)-pterocarpan from the (−)-isoflavanone could occur either via epimerization at the isoflavanone level, although there is at present no enzymological evidence to support this, or at the level of insertion of the 6a-hydroxyl group, which could conceivably involve the addition of water across a pterocarpene double bond, with subsequent inversion of stereochemistry. Arguments in favor of these two alternatives have been discussed in detail elsewhere[195] and the resolution of this problem requires more detailed enzymological studies.

1.28.3.10.4 Isoprenylation

At least half of the isoflavonoids that have been characterized from leguminous plants have side attachments derived from the 3,3-dimethylallyl (prenyl) group,[1] and several of these compounds have already been listed (**17–20, 26–28, 32, 80–82**). Further examples of prenylated isoflavonoids are given ((**88**)–(**92**)), from which the variability in position and complexity of prenylation, and also the subsequent metabolic modifications of the prenyl side chain, can be appreciated. Most of the enzymological work on isoflavonoid prenylation has been performed in relation to the elicitor/infection-induced prenylated pterocarpans in bean and soybean, or the constitutively expressed prenylated isoflavones in white lupin.

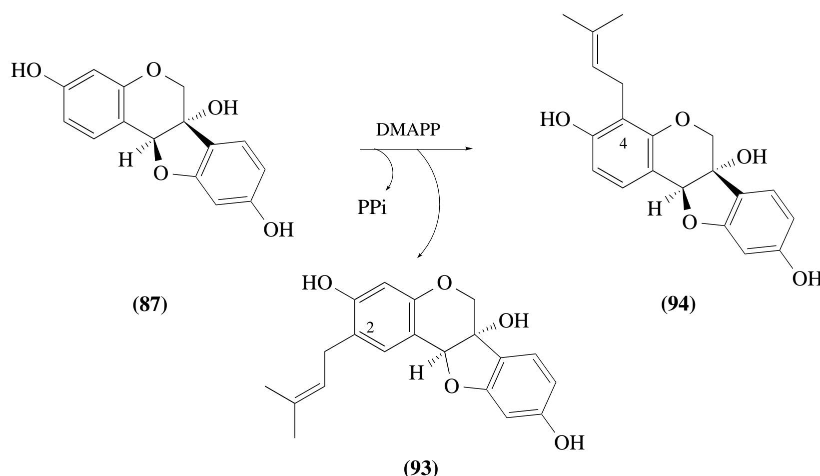
[^{14}C]Mevalonic acid is incorporated into the glyceollins (**18–20**) of soybean, and a cell-free preparation from elicited soybean cotyledons was shown to catalyze prenyl group addition from



(88) Striatine (*Mundulea striata*) **(89)** Erythbigenol A (*Piscidia erythrina*) **(90)** Erythbigenone A (*Piscidia erythrina*)

(91) Elliptone **(92)** Pomiferin

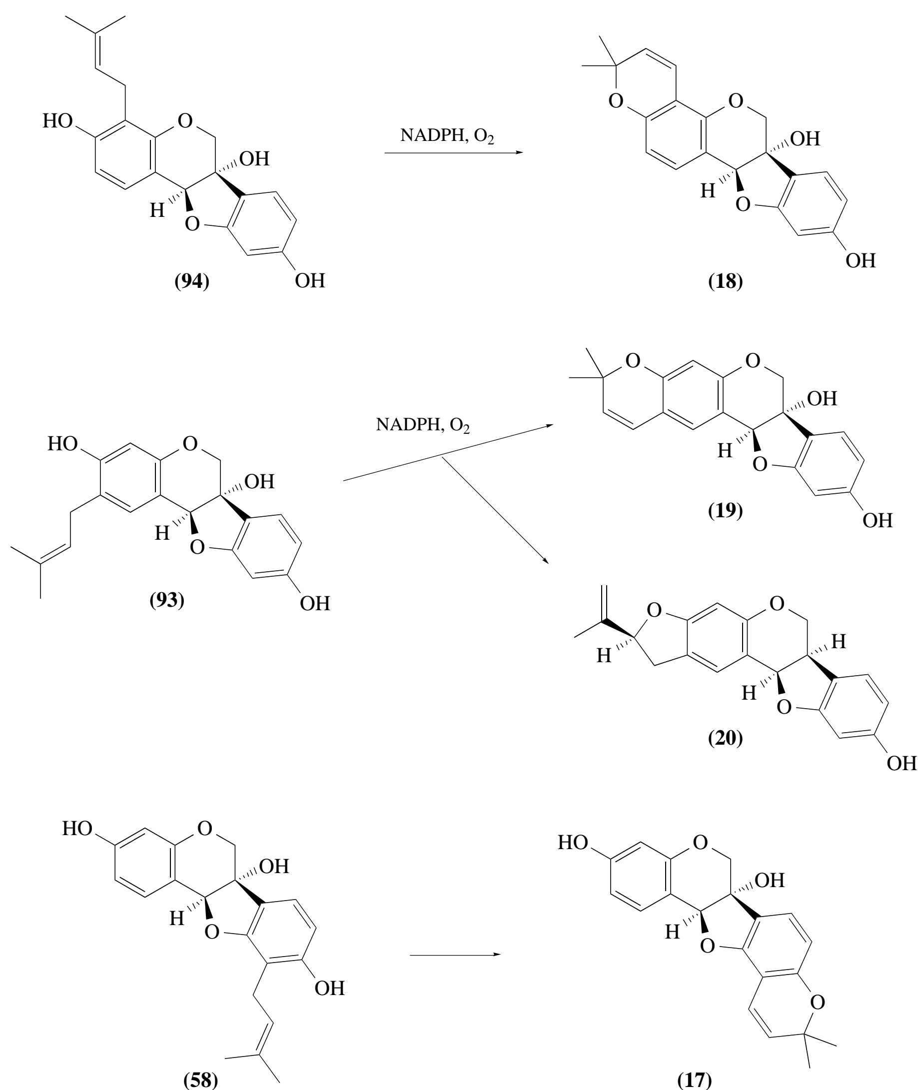
dimethylallyl pyrophosphate to the C-2 (major product (**93**)) and C-4 (minor product (**94**)) positions of 3,6a,9-trihydroxypterocarpan (**87**), as shown in Scheme 16.[217] The enzymatic activity appeared to be localized in a particulate fraction. Similarly, a microsomal fraction from yeast extract elicited bean (*Phaseolus vulgaris*) cell suspension cultures catalyzed the prenylation of 3,9-dihydroxy-pterocarpan (**57**) to 3,9-dihydroxy-10-dimethylallyl-pterocarpan (phaseollidin) (**58**). The K_m values were 1.5 μmol L^{-1} for DMAPP and 1.4 μmol L^{-1} (assuming that only the 6a*R*, 11a*R* enantiomer can act as substrate) for (**57**), and the enzyme was strongly product inhibited.[218] Sucrose- and Percoll-gradient centrifugation studies revealed that the pterocarpan prenyltransferases from both soybean and bean were localized to the chloroplast inner envelope membrane,[219] implying movement of isoflavonoid precursors among different cellular compartments.



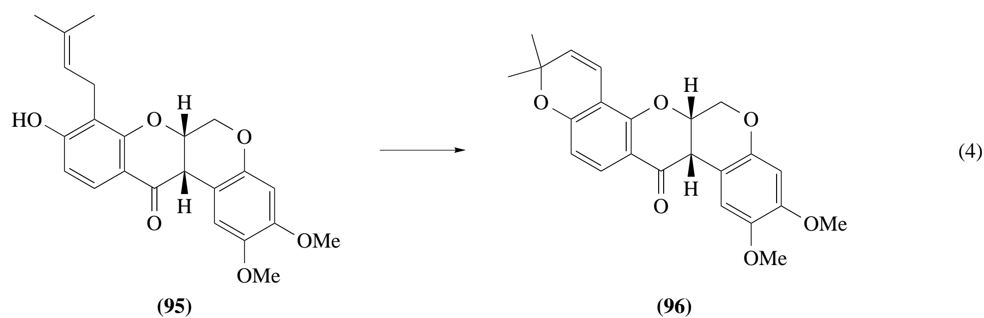
Scheme 16

The final stage in the biosynthesis of (**18–20**) in soybean and of phaseollin (**17**) in bean involves cyclization of the isoprene side chain (Scheme 17), and these reactions in soybean have been shown to be catalyzed by a cytochrome P450 monooxygenase system localized to the endoplasmic

reticulum.[220] As with the soybean prenyltransferase, cyclase activity is absent from unelicited cells and is strongly induced by exposure to elicitors from yeast or the fungal pathogen *Phytophthora megasperma* f. sp. *glycinea*.[220] The involvement of a membrane-bound cytochrome P450 in the cyclization of the prenyl group in the soybean pterocarpans contrasts with the demonstration of a soluble non-heme iron protein from *Tephrosia vogelii* that catalyzes oxidative ring closure of rot-2′-enoic acid (**95**) in the formation of the rotenoid deguelin (**96**)[221] (Equation (4)).



Scheme 17



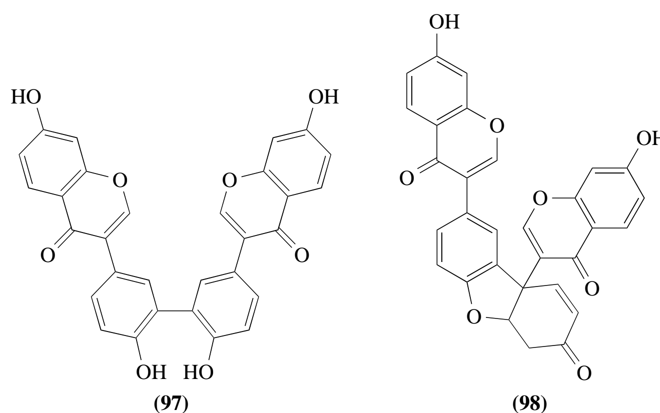
(4)

White lupin leaves, hypocotyls, roots, root nodules, and cell suspension cultures accumulate a range of prenylated isoflavones, and lupin microsomal enzyme systems have been described that catalyze the prenylation of genistein (**3**) and 2′-hydroxygenistein (**68**), from which these compounds are derived.[222,223] The prenyltransferase activity is constitutively expressed. The activity from radicles catalyzed prenylation of the two isoflavones at the 6-, 8-, and 3′-positions. Because different ratios of products were obtained with the enzyme preparation from different sources, and after different detergent solubilization steps, it was proposed that a number of distinct position-specific prenyltransferases may be present in lupin.[223]

Prenylation often increases the antifungal and anti-insect properties of isoflavonoids, and the value of introducing novel prenylated isoflavonoids into plants via genetic engineering was first proposed in 1987.[218] However, the isolation of the required prenyltransferase genes has been hampered by the difficulties inherent in the effective solubilization of the enzyme from the inner chloroplast membrane; although this has been achieved, recoveries are low.[223,224] It is to be hoped that molecular approaches such as differential library screening or PCR-based differential display will lead directly to the cloning of isoflavonoid-specific prenyltransferases, and that functional expression studies with different related sequences will then answer the outstanding questions concerning the molecular basis for substrate and position specificity.

1.28.3.10.5 Formation of isoflavone dimers

Dimeric isoflavonoids appear to be relatively rare in nature, with limited reports of the isolation and structural elucidation of such compounds as isoflavone–isoflavan, isoflavan–isoflavan, and isoflavone–isoflavone dimers.[1,225] Elicitation of cell cultures of *Pueraria lobata* results in the appearance of small quantities of kudzuisoflavones A and B (**97, 98**), dimeric isoflavones coupled through the B-rings.[225] As the latter is obtained as a racemic mixture, it is likely that these molecules arise via radical coupling, and may be viewed as artificial by-products of the action of peroxidase on daidzein (**4**).[225]

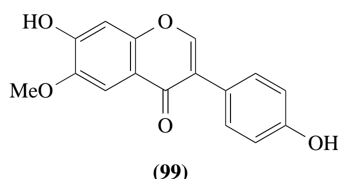


1.28.3.10.6 Formation and vacuolar storage and efflux of isoflavone glycosides

Isoflavonoids often occur constitutively as their glycosides and malonyl glycosides, and these compounds have generally been considered as "storage forms." The most studied systems for isoflavonoid glycoside metabolism are white lupin roots and cell cultures, soybean seeds and seedlings, and chickpea cell suspension cultures. Features of the accumulation and metabolism of these compounds differ somewhat in the different species.

In soybean seed hypocotyls, the 7-*O*-glucosides, 7-*O*-glucoside-6″-*O*-malonates, and 7-*O*-glucoside-6″-*O*-acetates of the isoflavones daidzein (**4**), genistein (**3**), and glycitein (**99**) occur, and have all been shown to increase during seed development in the pod, to maximum levels between 45 and 60 days after flowering.[226] Three days after germination, the metabolism of the young leaf shifts from isoflavonoid to flavonoid accumulation,[227] although low levels of isoflavone conjugates remain.[228] Cotyledons maintain high levels of conjugates of (**4**) and (**3**), but these same compounds decrease

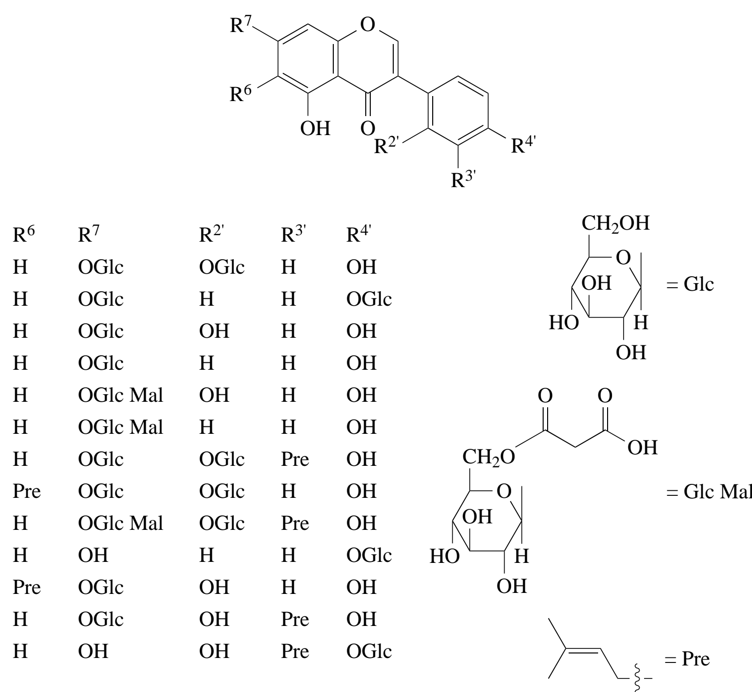
dramatically in the hypocotyl hook between 2 d and 4 d post-germination.[227] In soybean roots, conjugates of (**4**) predominate throughout development. A similar pattern of developmental distribution of isoflavonoids is observed in alfalfa, where formononetin 7-*O*-glucoside-6″-*O*-malonate (**34**) accumulates in roots, although in this case along with the malonyl glucoside of the pterocarpan medicarpin (**6**).[229]



(99)

Infection of soybean with *Phytophthora sojae* leads to dramatic changes in isoflavone glycoside profiles and distribution. Thus, in leaves, the pterocarpan glyceollin (**18**) accumulates to high levels only in the hypersensitive lesion formed in a resistant interaction, whereas the glucosides and malonyl glucosides of (**4**), (**3**), and (**99**) accumulate in a broad area around the lesion.[228] In cotyledons, the already large constitutive pools of isoflavone glycosides are rapidly mobilized in the incompatible (resistant) interaction with *P. sojae*, and, in the case of (**4**), the aglycone may be utilized for phytoalexin (glyceollin) synthesis.[230]

Hairy root and untransformed cell suspension cultures of white lupin (*Lupinus albus*) contain a range of mono- and diglucosides of (**3**), 2′-hydroxygenistein (**68**), and their 6- or 3′-prenyl derivatives[231–233] (Scheme 18; source references 111, 232 and 233). The same compounds are found in young plantlets, but unusually high levels are found constitutively in the hairy root and cell suspension cultures.[231,232] Although significant attention has been paid to the enzymology of isoflavone prenylation in lupin, nothing is known of the specificity of the enzyme system(s) required for 7-, 2′-, or 4′-*O*-glucosylation.

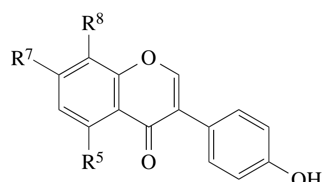


R^6	R^7	$R^{2'}$	$R^{3'}$	$R^{4'}$
H	OGlc	OGlc	H	OH
H	OGlc	H	H	OGlc
H	OGlc	OH	H	OH
H	OGlc	H	H	OH
H	OGlc Mal	OH	H	OH
H	OGlc Mal	H	H	OH
H	OGlc	OGlc	Pre	OH
Pre	OGlc	OGlc	H	OH
H	OGlc Mal	OGlc	Pre	OH
H	OH	H	H	OGlc
Pre	OGlc	OH	H	OH
H	OGlc	OH	Pre	OH
H	OH	OH	Pre	OGlc

Scheme 18

In cell suspension cultures of *Pueraria lobata*, (**4**) and (**3**) exist as their 6″-*O*-malonyl glucosides (7-*O*- and 8-*C*-glucosides for (**4**), 7-*O*-glucosides for (**3**)) (Scheme 19; source reference 234). Levels of the malonylglucosides rapidly decrease following exposure to yeast and fungal elicitors, or to hydrogen peroxide.[235,236] In elicitor-treated cells, the conjugates then reaccumulate, along with

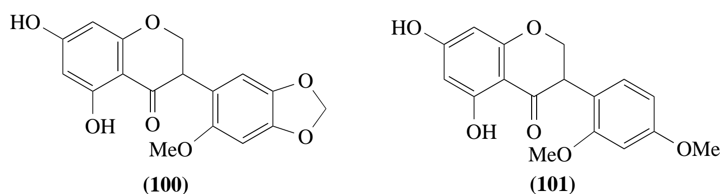
isoflavone aglycones and their dimers. The rapid decrease in conjugate levels, which correlates with accumulation of the derived aglycone into insoluble cell wall material, is unaffected by the protein synthesis inhibitor cycloheximide which does, however, prevent isoflavone reaccumulation, suggesting that metabolism of the conjugates utilizes preexisting enzymatic machinery.[235] A range of exogenously applied flavonoids and isoflavonoids were converted to the corresponding 7-*O*-glucoside-6″-*O*-malonates by the *Pueraria* cells. However, following elicitation, only the isoflavonoids were remobilized.[235] In alfalfa seedlings, elicitation with $CuCl_2$ leads to rapid mobilization of isoflavone conjugate stores, with resultant accumulation of (**6**) and formononetin (**5**).[237]



R5	R7	R8
H	OGlc	H
OH	OGlc	H
H	OH	Glc
OH	OH	Glc
H	OGlc Mal	H
OH	OGlc Mal	H
H	OH	Glc Mal
OH	OH	Glc Mal

Scheme 19

Chickpea cell suspension cultures have provided the most detailed information on the physiology and biosynthesis/catabolism of isoflavonoid glycosides. These cultures contain the malonyl glycosides of biochanin A (**33**), (**5**), cicerin (**100**), homoferreirin (**101**), (**6**), and maackiain (**59**), all of which are localized in the vacuole.[238] Treatment of the cells with an elicitor preparation from *Ascochyta rabiei* leads to accumulation of (**6**), synthesized *de novo* from L-phenylalanine. However, if the cells are elicited in the presence of the potent and specific phenylalanine ammonia-lyase (PAL) inhibitor L-α-aminooxy-β-phenylpropionic acid (AOPP), the vacuolar pool of (**34**) is mobilized as a precursor for medicarpin synthesis, indicating metabolic cross-talk between the central phenylpropanoid pathway and the pathway of isoflavone glycoside catabolism.[239] Furthermore, treatment of cells with low levels of yeast extract elicitor leads to acumulation of the malonyl glucosides of (**6**) and (**59**), whereas at high elicitor doses the pterocarpan aglycones accumulate, partially as a result of formation from mobilized conjugates.[240] Likewise, (**6**) is in part formed from mobilized (**34**), and from medicarpin malonyl glucoside, in elicitor-treated alfalfa cell suspenions.[241] The elicitor-mediated vacuolar efflux of isoflavone conjugates in chickpea is blocked by *trans*-cinnamic acid, the product of the PAL reaction,[242] providing a potential mechanism for how the mobilization machinery can ‘‘sense’’ the flux through the phenylpropanoid pathway.

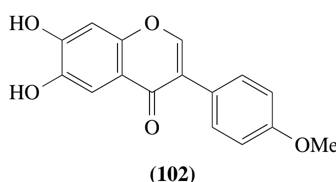


(100) **(101)**

The differential accumulation of isoflavonoid aglycones and glycosides as a function of elicitor concentration in chickpea cells is paralleled by changes in the activities of the enzymes of conjugate synthesis and catabolism. Thus, although increasing the elicitor concentration results in a proportional increase in the activities of early (PAL, C4H, CHS) and late (isoflavone 2′- and 3′-hydroxylase) enzymes for the synthesis of the isoflavonoid aglycones, the catabolic isoflavone

malonylesterase and glucosidase activities remain constant and then increase at the highest elicitor concentrations, whereas the glucosyltransferase and malonyltransferase of conjugate formation are highest at low elicitor concentrations, and then decrease.[240]

The isoflavone glucosyltransferase has been purified 120-fold from chickpea roots.[243] It is a cytoplasmic enzyme[238] of M_r 50 000, which preferentially glycosylates the 7-position of the 4′-*O*-methylated isoflavones (**5**) (K_m 24 μmol L^{-1}) and (**33**) (K_m 12 μmol L^{-1}). Compounds (**3**) and (**4**) are poor substrates, and 6,7-dihydroxy-4′-methoxyisoflavone (texasin) (**102**) is not glucosylated. A 47 kDa glucosyltransferase which acts on the A-ring hydroxyl groups of coumestrol (**9**) (K_m 57 μmol L^{-1}) and (**6**) (K_m 24 μmol L^{-1}) has been partially purified from alfalfa cell suspension cultures.[244] Little is known concerning the enzymology of isoflavone *C*-glycoside formation, as occurs in *Pueraria lobata*.

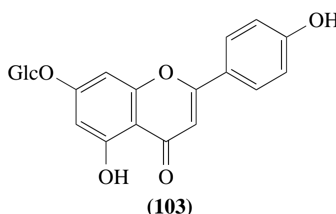


(102)

The isoflavone glucoside malonyl transferase also appears to be a cytoplasmic enzyme in chickpea.[238] It has an M_r of 112 000 and specifically malonylates the 6-position of the glucose residues of isoflavone 7-*O*-glucosides.[245] Its substrates, in order of decreasing activity, are the glucosides of (**33**), (**5**), (**3**), pratensin (**71**), (**4**), and (**59**). The enzyme does not act on 4′-*O*-glucosides.

The first stage of isoflavone conjugate mobilization appears to be catalyzed by an isoflavone glucoside malonylesterase that is vacuolar-localized in chickpea.[238] The enzyme has been purified over 700-fold from chickpea roots,[247] and possesses some unusual properties. The subunit M_r is ~32 kDa, although the holoenzyme M_r appears to be in excess of 2×10^6. The enzyme has very little activity against standard non-specific esterase substrates, and is unaffected by standard esterase inhibitors. It has a high affinity for biochanin A 7-*O*-glucoside-6″-*O*-malonate (K_m 4.4 μmol L^{-1}).

As most β-glucosidases lack absolute specificity for any particular substrate, it is often difficult to know their true metabolic functions. Chickpea roots contain three isoforms of β-glucosidase that are only found in the isoflavonoid-containing tissues of the plant.[247] The enzymes are dimers of M_r ≈ 130 000, and have low K_m (20–40 μmol L^{-1}) and high V_{max} values for the 7-*O*-β-glucosides of (**5**) and (**33**), although apigenin 7-*O*-β-glucoside (**103**) is an equally good substrate. In contrast to the malonylesterase, isoflavone β-glucosidase activity appears to be primarily cytoplasmic.[238]



(103)

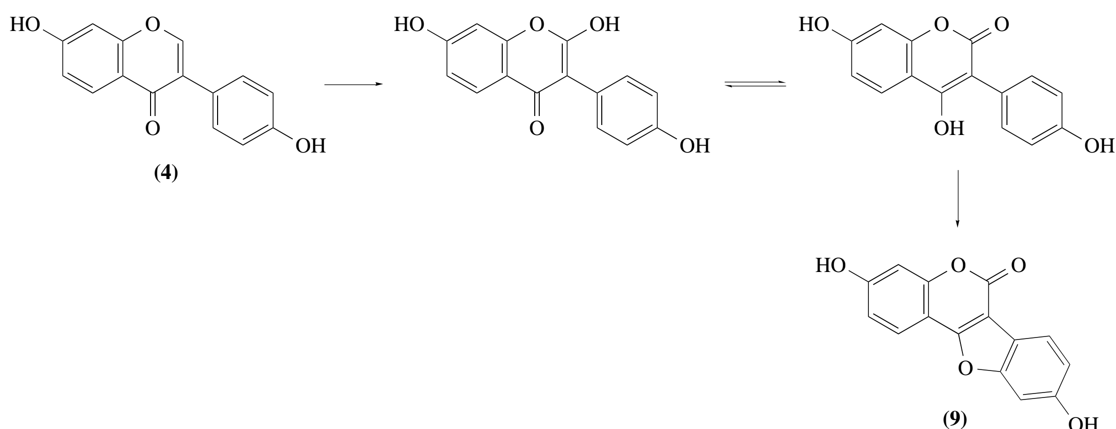
Unconjugated medicarpin (**6**) is taken up into isolated vacuoles of mung bean (*Vigna radiata*) at a low rate in the presence or absence of MgATP. However, following incubation of [^{3}H]medicarpin and glutathione with a maize glutathione *S*-transferase preparation, the resultant [^{3}H]medicarpin–glutathione conjugate(s) is taken up eight times faster in the presence than in the absence of MgATP.[248] Uptake of medicarpin–glutathione is not significantly inhibited by the protonophore gramicidin-D, but is strongly inhibited by vanadate and the alternative transport substrate *S*-(2,4-dinitrophenyl)glutathione.[248] These results demonstrate the operation of the high-affinity, high-capacity, glutathione conjugate (GS-X) pump, previously shown to be involved in the vacuolar uptake of xenobiotics[249] and anthocyanin,[250] in the vacuolar transport of an isoflavonoid phytoalexin.

Because glycosylated anthocyanin is a substrate for the GS-X pump[250] the question remains as to whether medicarpin conjugates, in addition to the aglycone, are also substrates for the vacuolar GS-X pump. It is therefore important to clarify whether glycosylation and malonylation of isoflavonoids occur in the cytoplasm, the vacuole, or both, to determine how glutathione is removed

from conjugates, and, most importantly, to understand what determines whether a vacuolar compound will be permanently or temporarily sequestered. Answers to these questions with respect to isoflavonoids will provide clues as to how the host plant integrates the balance between attack and defense during the determinative stages of pathogen infection. It is interesting to speculate that one function for the GST(s) induced following the hypersensitive response to avirulent fungal pathogens[116,251] could be in facilitating the vacuolar storage of antimicrobial isoflavonoids in the healthy cells around the hypersensitive lesion.

1.28.3.11 Biosynthesis of Coumestans

An early hypothesis proposed that coumestrol (**9**) might be formed from daidzein (**4**) by hydroxylation at the 2-position, followed by tautomerization and ring closure, as shown in Scheme 20. Subsequent ^{14}C-labeling studies revealed good incorporation of (**4**), 2′,4′,7-trihydroxyisoflavone (**55**) and dihydrodaidzein (**104**) into (**9**) in mung bean seedlings, leading to the proposal that the pathway operated via a metabolic grid leading to a pterocarp-6a-ene intermediate (**105**) (Scheme 21).[252,253] In $CuCl_2$-treated alfalfa seedlings, in which accumulation of 9-*O*-methylcoumestrol (**10**) was induced with little effect on the level of (**9**), (**4**) was incorporated into (**9**) but not into (**10**), although formononetin (**5**) was incorporated into the latter.[130] This labelling pattern reflects that discussed in Section 1.28.3.10.1 in relation to the paradox concerning the origin of the 4′-*O*-methyl group in isoflavones destined for pterocarpan synthesis.

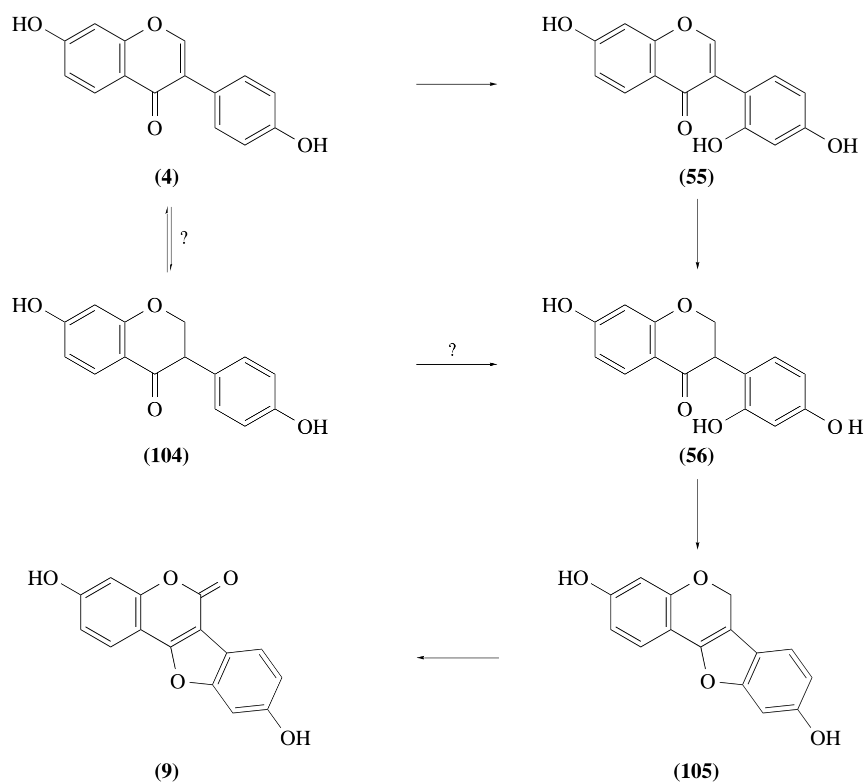


Scheme 20

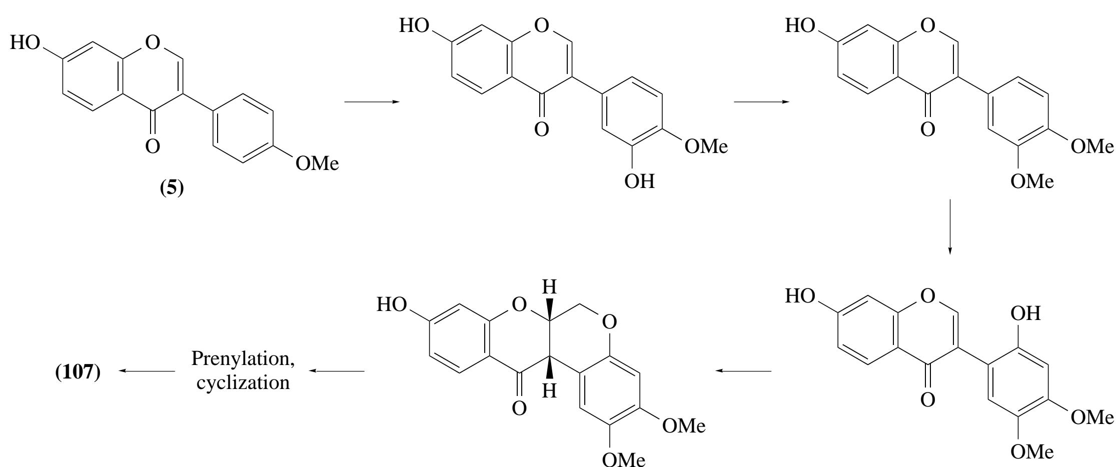
7,2′-Dihydroxy-4′-methoxyisoflav-3-ene (**46**) and 7,2′-dihydroxy-4′-methoxyisoflav-3-ene-2-one (**47**) were excellent precursors of (**10**) in elicited alfalfa seedlings, leading to the proposal of a pathway to coumestans involving the intermediacy of isoflav-3-enes and 3-arylcoumarins (Scheme 4).[131] To the best of the author's knowledge, the enzymology of these reactions remains to be worked out.

1.28.3.12 Biosynthesis of Rotenoids

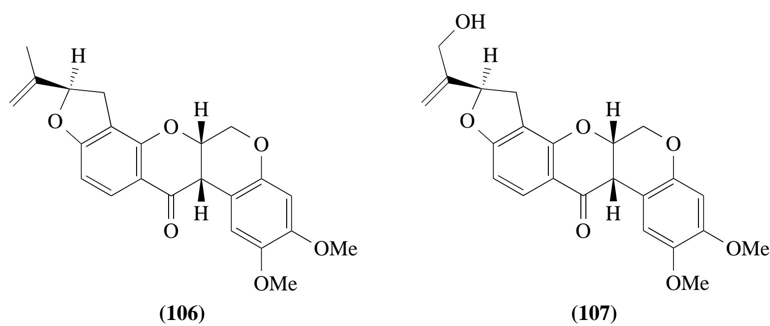
The rotenoids are characterized by their four-ring chromanochromanone structure. Biosynthetically, the carbon skeleton can be envisaged as arising from an isoflavanone with an extra carbon atom that could arise initially as a methoxyl group at the 2′-position (isoflavanone numbering), yielding the position 6 carbon of rotenoids. This basic scheme is supported by the results of radiolabeled precursor studies in seedlings of *Amorpha fruticosa*, which accumulate rotenone (**106**) and amorphigenin (**107**).[254] In the case of (**107**), it appears that formononetin (**5**) is first hydroxylated at the 3′-position, and methylation of this position may then occur prior to substitution of the 2′-position and subsequent closure of ring B via conjugate addition of a methoxyl radical (Scheme 20).



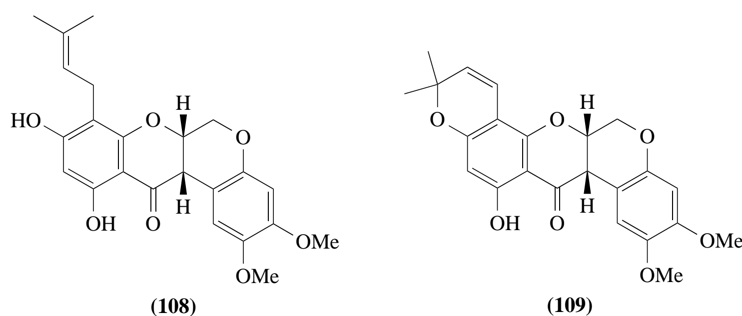
Scheme 21



Scheme 22



Prenylation and subsequent prenyl cyclization are late steps in rotenoid biosynthesis. Seeds, plants, and plant cell cultures of the West African tropical plant *Tephrosia vogelii* have been used to study the formation of the chromen ring that occurs in the rotenoid deguelin (**96**).[221,255] A soluble enzyme, deguelin cyclase, was isolated and partially purified. The cyclase catalyzes the direct formation of (**96**) from rot-2′-enoic acid (**95**) (Equation (4)) without the appearance of a hydroxylated intermediate.[221] The enzyme, which is inhibited by metal chelators, utilizes molecular oxygen, requires no cofactors, and has a K_m value for (**95**) of 4.6 μmol L^{-1} and an M_r of ~39 000. It also catalyzes the conversion of sumatrolic acid (**108**) to α-toxicarol (**109**), but does not convert (**95**) to (**106**). The stereochemistry of the reaction has been determined[256] and a reaction mechanism proposed that parallels a suggested mechanism for the formation of (**106**) from (**95**).[256] Deguelin cyclase is clearly not a cytochrome P450, and is therefore distinct from the enzyme(s) catalyzing prenyl to chromen transformations in the pterocarpans.[220]

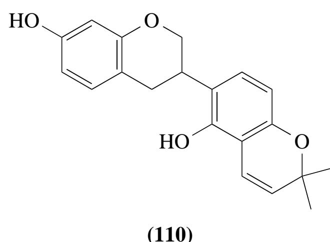


1.28.4 CATABOLISM OF ISOFLAVONOIDS

1.28.4.1 Metabolism by Plant Tissues

Isoflavonoids may not be end products of plant metabolism. In addition to demonstrating their mobilization from vacuolar stores and subsequent metabolism (often to more highly modified isoflavonoid derivatives, see above), some studies have documented metabolism of endogenously applied isoflavonoids by plant tissue. However, the presence of contaminating microorganisms can seriously compromise the interpretation of such experiments. For example, studies with chickpea and mungbean seedlings indicated half lives for exogenously added daidzein (**4**), formononetin (**5**) or coumestrol (**9**) of ~50 h. However, repeating these experiments with sterile mung bean seedlings revealed little appreciable metabolism of (**5**) (95% recovery after 24 h), although [^{14}C]-(**4**) was rapidly metabolized (8.5% recovery) with label incorporated into most cellular/chemical fractions, including the cell wall.[257]

The interconversions of medicarpin (**6**) and its corresponding isoflavan vestitol (**8**) in alfalfa and red clover[102,103] have been described above. Ring opening of a pterocarpan to yield the corresponding isoflavan (**110**) has also been reported when phaseollin (**17**) is fed to bean cell suspension cultures,[258] and this is accompanied by the opening of the ring formed from the cyclized prenyl side chain. Compound (**17**) is also converted to (**110**) by the fungal pathogen *Septoria nodorum*.[259]



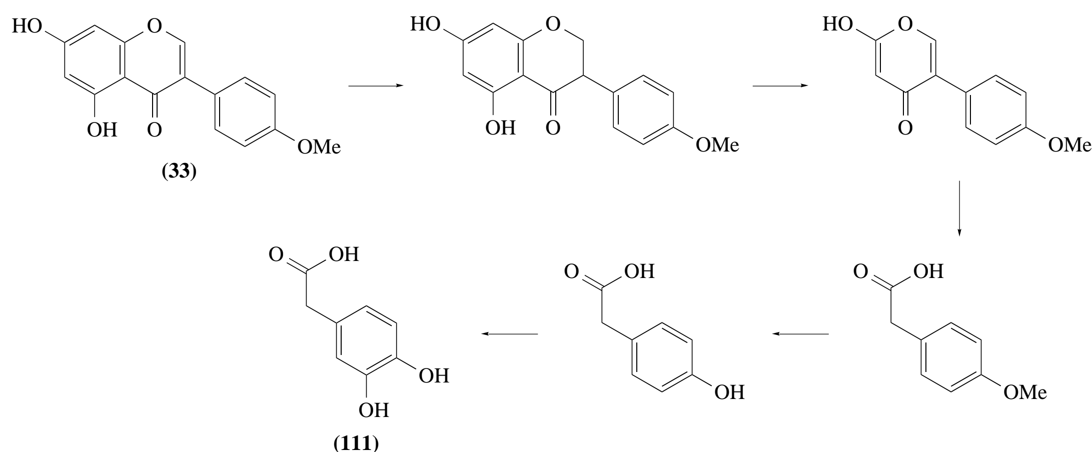
The role of isoflavonoid degradation as a factor in the elicitor- and pathogen-induced accumulation of isoflavonoid phytoalexins received considerable attention when it was proposed that elicitation by abiotic elicitors or incompatible races of pathogens was associated with strongly inhibited phytoalexin degradation (assessed using exogenously applied radiolabeled phytoalexin),

whereas an increased biosynthetic rate was the major factor determining phytoalexin levels in response to biotic elicitors.[260,261] These conclusions were challenged when it was demonstrated, using $^{14}CO_2$ labeling *in vivo*, that the half-lives of glyceollin (**18**) and its trihydroxypterocarpan precursor (**87**) were long, ~100 h and ~38 h, respectively.[262] Apparently, the metabolic fates of exogenously applied and endogenously synthesized glyceollin are different. Studies of isoflavonoid turnover have subsequently been eclipsed by the vast body of work on the induced biosynthesis of these compounds, and more studies are needed to determine the biological half-lives and metabolic fates *in planta* of biologically active isoflavonoids.

1.28.4.2 Metabolism by Plant Pathogenic Fungi

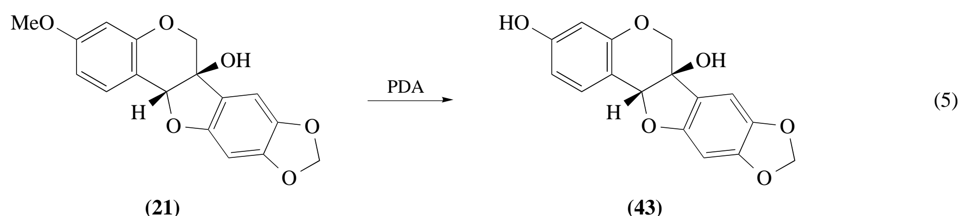
There is a considerable body of literature on the metabolism of isoflavonoids by phytopathogenic fungi. A driving force for much of this work is the observation that metabolism of isoflavonoid phytoalexins, leading to their detoxification, is a mechanism by which successful pathogens may overcome the resistance response of their host.[9] The following discussion outlines metabolic strategies for phytoalexin detoxification for a limited selection of compounds, concentrating on pathways for which molecular genetic and/or enzymological data are available. This topic has been reviewed in more detail elsewhere.[263]

The simple isoflavone biochanin A (**33**) is degraded by *Nectria haematocca* to 3,4-dihydroxyphenylacetic acid (**111**) (Scheme 23),[264] and similar metabolic products have been obtained with other related fungi. The pathway involves sequential fission of the A-ring and the heterocyclic ring.



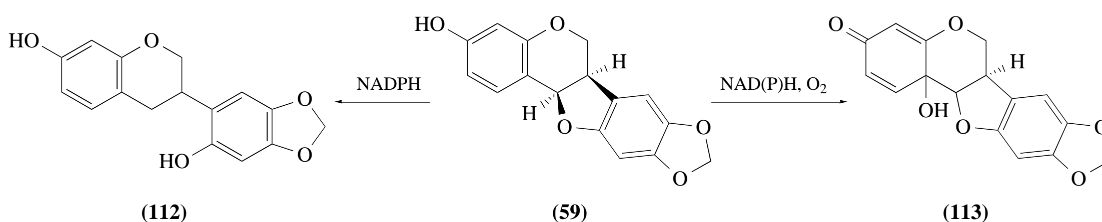
Scheme 23

The pea phytoalexin pisatin (**21**) is detoxified by *N. haematococca* by demethylation at position 3 (Equation (5)).[265] The enzyme that catalyzes this reaction, pisatin demethylase (PDA), is a cytochrome P450 that has been studied extensively at both the biochemical[266] and genetic[9,267] levels. Several different *PDA* genes are present in the *N. haematocca* genome, and their expression levels appear to confer different levels of demethylating activity.[268] The *PDA*6 gene is localized on a small meiotically unstable chromosome that is dispensable for normal growth.[269] The *PDA*1 and *PDA*9 genes contain an upstream sequence that is important for the induction of the genes by (**21**),[270,271] and this element is absent from *PDA*6. The sequences responsible for induction of *PDA*1 by (**21**), and the transcription factors that bind to these elements, have been studied in detail.[272–275]



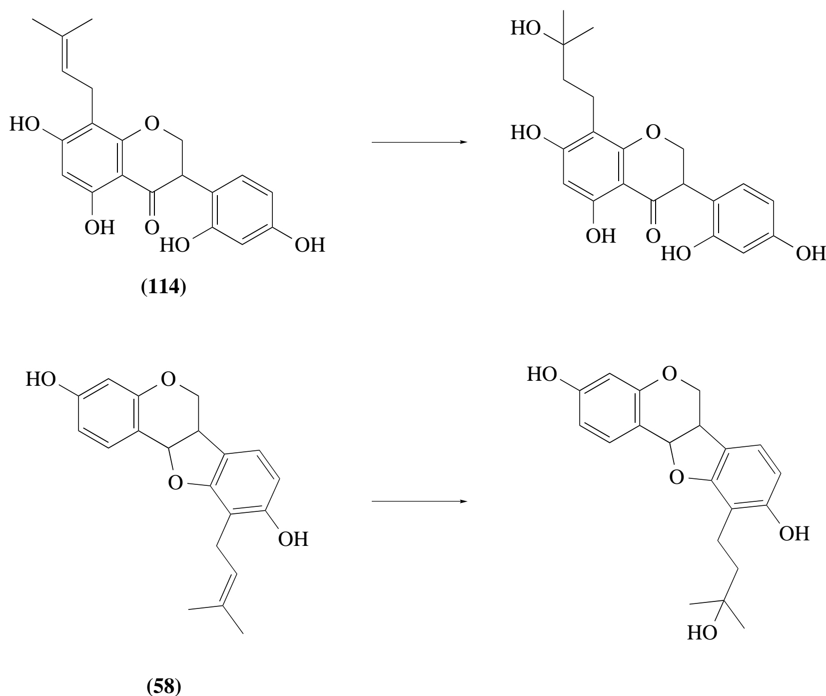
(5)

Ascochyta rabiei converts the chickpea pterocarpan phytoalexin (−)-maackiain (**59**) to a range of catabolites via reduction to 2′-hydroxyisoflavan (**112**) or oxidation to a 1a-hydroxypterocarp-1,4-dien-3-one (**113**)[276] (Scheme 24). The latter reaction has also been observed during the metabolism of phaseollin (**17**) by *Fusarium solani*.[277] A flavoprotein monooxygenase catalyzing 1a-hydroxylation of (**59**) in the presence of NAD(P)H, FAD, and molecular oxygen, and a pterocarpan reductase catalyzing the conversion of pterocarpan to 2′-hydroxyisoflavan (**112**) in the presence of NADPH, have been purified and characterized from *A. rabiei*[278,279] in which they are constitutively expressed. Three genes involved in maackiain detoxification by *N. haematococca*, which occurs via 1a- or 6a-hydroxylation, have been characterized. The *MAK*1 gene which, like *PDA*6, is located on a dispensable chromosome, appears, from nucleotide-derived amino acid sequence data, to encode a soluble flavoprotein monooxygenase,[280] similar to the pterocarpan hydroxylase described from *A. rabiei*.[278]



Scheme 24

Fusarium solani f. sp. *phaseoli* detoxifies the bean phytoalexins kievitone (**114**) and phaseollidin (**58**) by hydration of the prenyl side chain[281,282] (Scheme 25). The enzyme kievitone hydratase has been purified from *F. solani*, and its gene cloned.[283,284] The protein is extensively glycosylated, and is encoded by a single locus in *F. solani*.

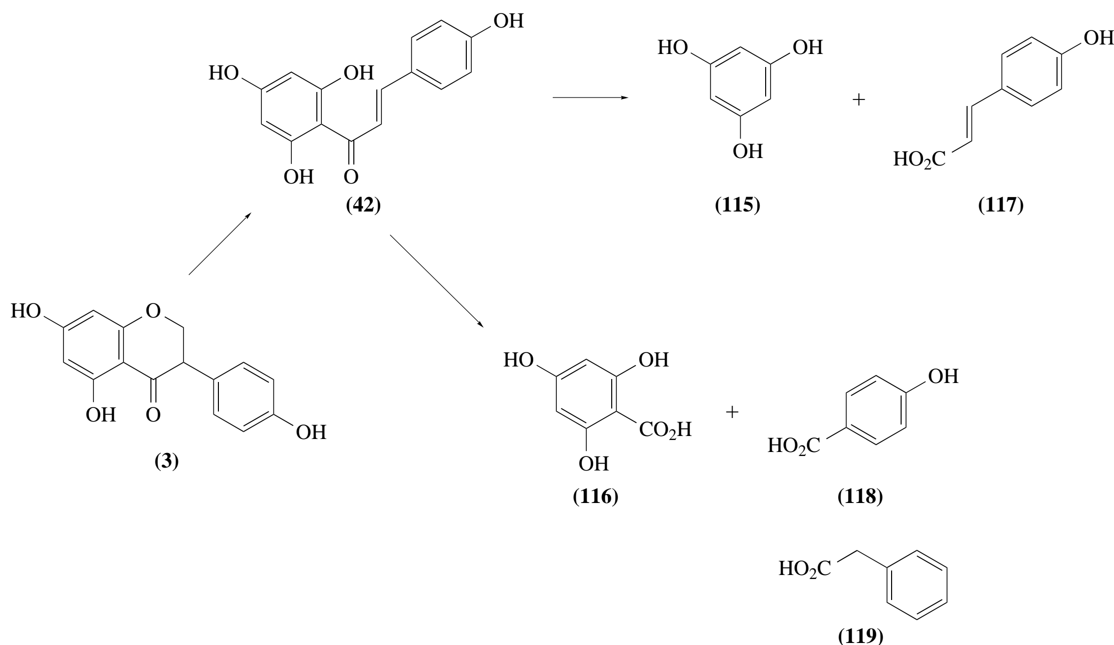


Scheme 25

1.28.4.3 Metabolism by Bacteria

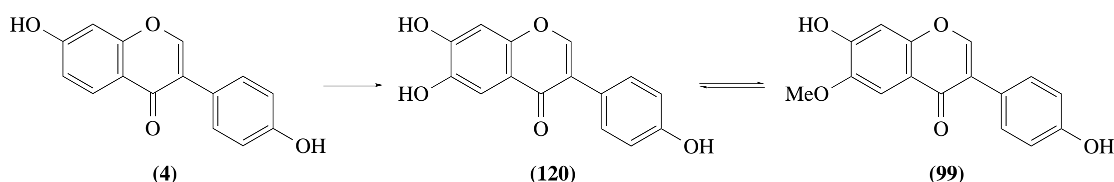
Isoflavonoid *nod* gene inducers can be metabolized by *Rhizobia* to a number of simpler compounds. Thus, genistein (**3**) is converted to naringenin chalcone (**42**), which is then converted

to phloroglucinol (**115**), phloroglucinol carboxylic acid (**116**), 4-coumarate (**117**), *p*-hydroxybenzoic acid (**118**), and phenylacetic acid (**119**) (Scheme 26).[285] The degradative reactions generally involve C-ring fission, and coumestrol (**9**) is produced as a C-ring metabolite of daidzein (**4**).[286] Some of the metabolic products are themselves effective *nod* gene inhibitors.



Scheme 26

Interest has been shown in the bacterial metabolism of soybean isoflavones in relation to the occurrence of isoflavone metabolites in tempe, a fermented soybean food from Indonesia. One important metabolite is 6,7,4′-trihydroxyisoflavone (**120**), which has powerful antioxidant activity. *Brevibacterium epidermidis* and *Micrococcus luteus* demethylate the soybean isoflavone glycitein (**99**) to (**120**), whereas *Microbacterium arborescens* was shown to convert (**4**) to (**99**) via (**120**) (Scheme 27).[287]

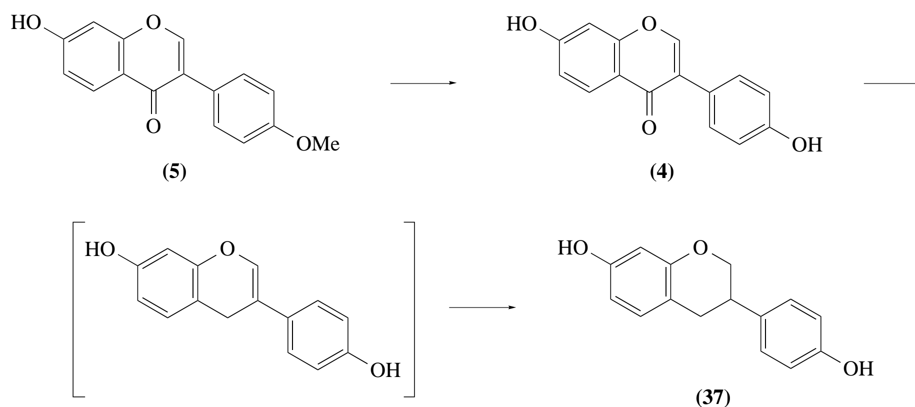


Scheme 27

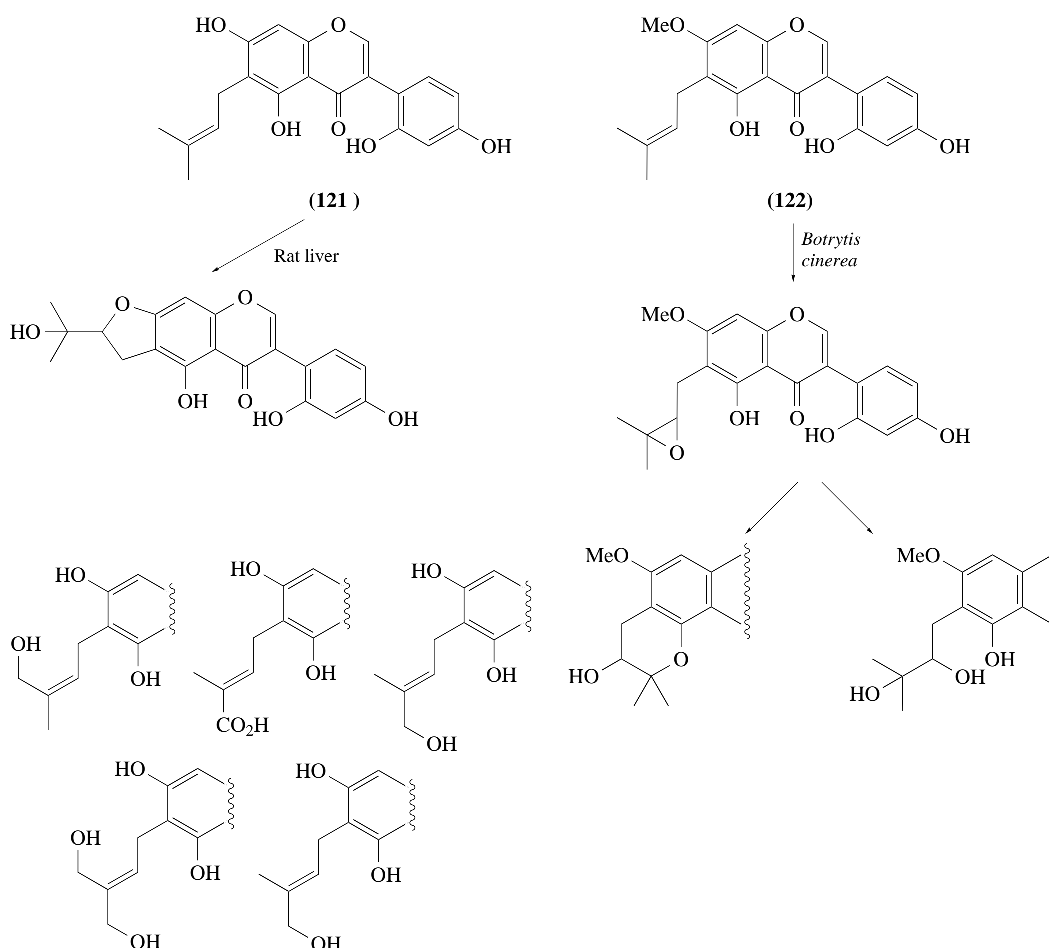
1.28.4.4 Metabolism by Animals

The conversion of dietary soybean isoflavones to urinary equol (**37**) has already been referred to. This is catalyzed by gastrointestinal flora. The proposed pathway[97] is summarized in Scheme 28.

Studies have been performed on the metabolism of the prenylated lupin isoflavone luteone (**121**) in a rat-liver homogenate.[288] All the interconversions involved side-chain oxidation reactions, most of which were distinct from those observed following incubation of 7-methylluteone (**122**) with *Botrytis cinerea*[289,290] (Scheme 29). The enzymology of the side chain oxidation in rat liver homogenates has yet to be determined. In *B. cinerea*, formation of the epoxide is catalyzed by a microsomal monooxygenase that is inducible by prenylated isoflavones.[290]



Scheme 28



Scheme 29

1.28.5 INTEGRATED CONTROL OF ISOFLAVONOID BIOSYNTHESIS

In elicited plant cells, the accumulation of isoflavonoids is usually accompanied by coordinated increases in the extractable activities of all the biosynthetic enzymes of the pathway. These increases are primarily the result of increased transcription of the genes encoding the various biosynthetic

enzymes,[146,159] and the evidence for increased gene transcription has already been described above in relation to those enzymes for which molecular probes are available.

Two major questions are now attracting increased attention: what are the signal transduction pathways linking elicitor perception at the cell surface to increased transcription of phytoalexin biosynthetic genes, and how are these integrated within the total program of induced defense responses? A related question is, how many different transcriptional activators are necessary to orchestrate the complete pathway response? To address these questions, it is necessary to isolate the transcriptional regulators responsible for the elicitation response. Because of the ease of selection for mutations affecting the synthesis of colored flavonoid derivatives, genetic approaches have been successfully used for the cloning of transcriptional regulators of the anthocyanin pathway.[291] Such a strategy is much less easy for colorless isoflavonoids, and the genetic intractability of many of the legume species used for isoflavonoid research is a further problem. The development of model genetic systems in legumes such as *Medicago truncatula*[292] provides hope that it will soon be possible to use mutation followed by positional cloning to isolate genes involved in the regulation of isoflavonoid synthesis.

The alternative approach is to use a combination of molecular and biochemical techniques to identify the *cis*-elements in isoflavonoid pathway gene promoters that confer infection- or elicitor-inducibility,[293,294] to use this information to generate probes for the isolation of the transcription factors that bind to these sequences, and then to search for molecules that might interact with these factors to modulate their activity. The author's group have performed such a series of experiments using an elicitor-inducible *CHS* gene promoter as the starting point for walking back up the signal transduction pathway for elicitor modulation of isoflavonoid synthesis.[146,295] At least two distinct classes of DNA-binding protein interact with the elicitor-response transducing element in the bean *chs*15 promoter, and their activity is regulated by a highly poised phosphorylation/dephosporylation cascade.[296] Several reviews give detailed background on the status of our understanding of defense gene signal transduction pathways.[298,299]

Transcription of the *IFR* gene is slightly delayed compared with that of *PAL* or *CHS* genes in elicited alfalfa cell suspensions, consistent with its responding to different transcriptional regulators.[159] This idea is confirmed by the results of *in vivo* (functional) and *in vitro* analyses of the alfalfa isoflavone reductase promoter,[297] which appears to be regulated by transcription factor(s) recognizing sequences not involved in CHS regulation.

1.28.6 EVOLUTION OF ISOFLAVONOID PHYTOALEXIN BIOSYNTHETIC PATHWAYS

Genes encoding enzymes of plant natural product biosynthesis have most likely arisen from duplication and subsequent mutation of genes encoding enzymes of primary metabolism. Natural selection will act to preserve such altered gene sequences if they confer a selective advantage, i.e., if some new functionality is produced. It has been argued that the original function leading to selection for flavonoid pigments was their ability to modulate internal growth regulator levels (e.g., by stimulating or inhibiting IAA oxidation), because this could be manifest at relatively low levels of product present in the cytoplasm.[300] UV-filtering vacuolar flavonoid pigments would have evolved later.

In view of their increased complexity and limited taxonomic distribution compared with 1,3-diphenylpropane flavonoids, it is clear that the isoflavonoids evolved more recently than the UV-protective flavonoids. The selective advantage of the first isoflavonoids could have been as antimicrobial agents. Thus, microbial infection would be a strong selection pressure for somatic mutations occurring even in a single branch or shoot that could give rise to protected tissue, as argued by Walbot.[301] However, simple isoflavonoids generally have low antimicrobial activity, and it is unlikely that the position-specific modifying enzymes that generate highly bioactive isoflavonoids would have arisen in parallel with the isoflavone synthase system. Other functions for the earliest isoflavonoids must therefore be sought, and the evidence outlined below suggests that antioxidant activity is a likely possibility.

Many flavonoids, and also lignans and hydroxycinnamic acid derivatives, have powerful antioxidant activity,[302] and can scavenge superoxide anion,[303] or help remove apoplastic hydrogen peroxide by acting as cosubstrates for ascorbate-dependent peroxidases.[304] These active oxygen species are produced during the oxidative burst, a key component of plant disease resistance responses that has many common features with mammalian neutrophil activation,[294] and is probably therefore of ancient evolutionary origin. Using a screen that selects for plant cDNA clones that can

confer oxidative stress tolerance when expressed in yeast, an Arabidopsis NADPH reductase was characterized that has striking amino acid sequence similarity to alfalfa IFR,[305] and further IFR homologues have been reported from the Arabidopsis expressed sequence tag (EST) project.[306] A similar IFR-like protein is induced in maize by treaments that affect redox balance by reducing cellular glutathione levels.[307] Neither the Arabidopsis nor maize proteins exhibit IFR activity, and neither plant has been reported to make isoflavonoids; the proteins appear to belong to a new class of oxidoreductases that may function in a thiol-independent response to oxidative stress under conditions of reduced glutathione shortage.

Other enzymes with very strong amino acid sequence identity to IFR have been identified in tobacco floral, stem, and root tissue,[308,309] but are clearly not involved in isoflavonoid synthesis. The gene encoding the IFR-like protein that is expressed in tobacco flowers and stems has several introns that are in the identical position to the introns in the alfalfa IFR gene,[196] indicating close evolutionary relatedness. The functions of the tobacco IFR-related proteins remain to be determined.

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