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Article:
Dixon, R.A. (2004) Phytoestrogens. Annual Review of Plant Biology. pp. 225-261. ISSN: 1543-5008

https://doi.org/10.1146/annurev.arplant.55.031903.141729

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Annu. Rev. Plant Biol. 2004. 55:225–61
doi: 10.1146/annurev.arplant.55.031903.141729
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First published online as a Review in Advance on January 7, 2004

PHYTOESTROGENS

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Key Words isoflavone, lignan, soybean, flaxseed, nutraceutical

■ **Abstract** Collectively, plants contain several different families of natural products among which are compounds with weak estrogenic or antiestrogenic activity toward mammals. These compounds, termed phytoestrogens, include certain isoflavonoids, flavonoids, stilbenes, and lignans. The best-studied dietary phytoestrogens are the soy isoflavones and the flaxseed lignans. Their perceived health beneficial properties extend beyond hormone-dependent breast and prostate cancers and osteoporosis to include cognitive function, cardiovascular disease, immunity and inflammation, and reproduction and fertility. In the future, metabolic engineering of plants could generate novel and exquisitely controlled dietary sources with which to better assess the potential health beneficial effects of phytoestrogens.

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INTRODUCTION

In addition to being a source of compounds necessary for human nutrition, certain plant foods also contain compounds that may have long-term effects on human and animal health. Among the most important are the phytoestrogens. The term phytoestrogen first appeared in the literature in the late 1980s, and phytoestrogens have since become a major topic of research, as shown by the numbers of citations including the term listed in biological abstracts. The numbers parallel the use of the term nutraceutical (Figure 1). Most of the recent citations on phytoestrogens address potential health promotion in mammalian systems. Two of the most important and potent phytoestrogens, equol and enterolactone, do not accumulate in plants but are microbial degradation products of the soy isoflavone daidzein and the flaxseed lignans secoisolariciresinol and matairesinol, respectively, formed in the gut (165, 191). As shown in Figure 1, the potential estrogenic effects of lignans were realized some time later than those of isoflavones, and thus a significantly larger percentage of the research reports on lignans are nonhealth related compared with those on isoflavones.

Estrogenic compounds appear in more than 300 plant species, but few of these are consumed by animals or humans. Some plants contain steroidal estrogens,

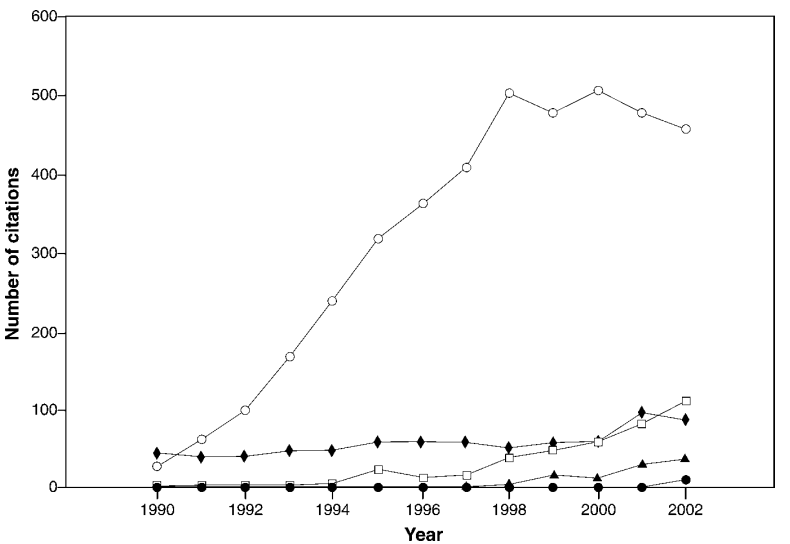


Figure 1 Citation statistics for papers relating to phytoestrogens. Graph shows the number of citations per year from 1990 to 2002, with various terms appearing in the titles or abstracts, or as key words. The numbers were obtained from searching biological abstracts. The symbols on the graph represent annual citations for the terms genistein (○), phytoestrogen (□), lignan (♦), nutraceutical (▲), and phytoestrogen plus lignan (●).

but as these are essentially based on the same structures that occur naturally in animals, they are not considered phytoestrogens by the strictest definition (3). The common phytosterols such as β-sitosterol, campesterol, and stigmasterol do not bind to human estrogen receptors (ER) and do not exert estrogenicity in female rats (15). Therefore, we do not further discuss plant sterols here. This leaves the soy isoflavones genistein and daidzein, the chickpea isoflavone biochanin A, the clover isoflavone formononetin, the isoflavonoid-derived coumestan coumestrol, and the flaxseed lignans as the major phytoestrogens relevant for human and animal health (Figure 2, see color insert; Figure 3). Almost all these compounds occur in the plant in glycosylated forms, and importantly, the bioavailability of the glycoconjugates might be different from that of the unsubstituted aglycones, which are often used in animal feeding and model cell culture studies.

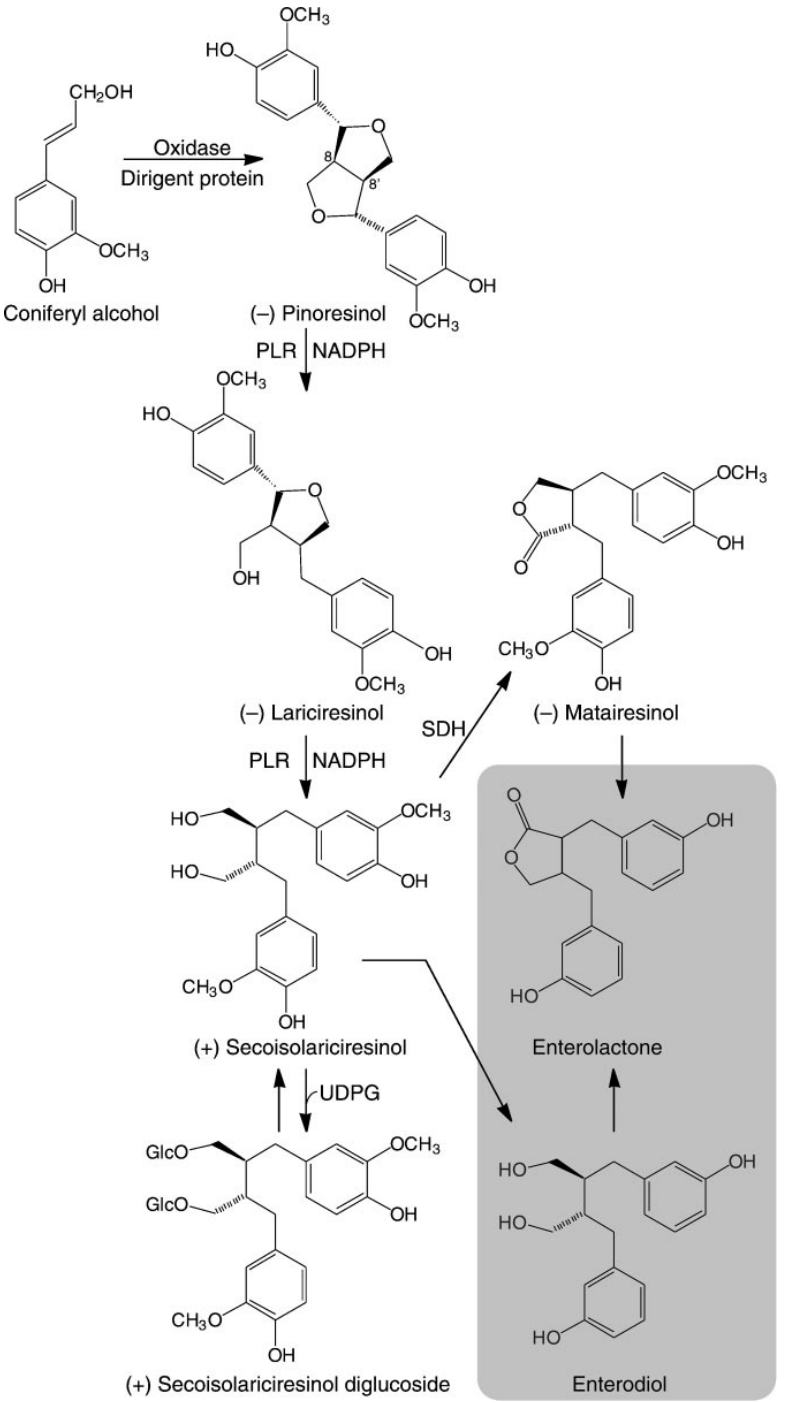
There are several excellent reviews documenting and evaluating the large and complex body of evidence purporting to demonstrate the health beneficial effects of phytoestrogens for humans and laboratory animals (2–4, 7, 166). This review departs from the main focus of these articles by describing recent advances in our understanding of the potential health benefits of dietary phytoestrogens and by including the biochemistry of phytoestrogens in the plant.

ISOFLAVONOID PHYTOESTROGENS

Occurrence and Dietary Sources

The isoflavonoids enjoy a restricted distribution in the plant kingdom, being mostly limited to the subfamily Papilionoideae of the Leguminosae (44). The first evidence that plants contained estrogenic compounds came from investigations on the negative effects of clovers on fertility in cattle and sheep, reviewed in (163). Breeding to reduce the isoflavone phytoestrogen formononetin (Figure 2*B*) became an important goal, and new high-yielding clover varieties with reduced formononetin levels were recently released (23). Isoflavone phytoestrogens may also affect avian fertility. In 1976 it was hypothesized that California quail switched their feeding preference to legumes containing formononetin to control (reduce) their fertility at times of food shortage (114).

Isoflavonoids are derived directly from flavanones, compounds ubiquitously present in plants. They differ from the flavonoids in having the B-ring linked to the 3- rather than the 2-position of the central heterocycle (see Figure 2*B*). The overall structural variation among isoflavonoids is surprisingly large, involving not only the number and complexity of substituents, but also different oxidation levels of the heterocycle and even the presence of additional heterocyclic rings, usually methylenedioxy (formed by cyclization between vicinal hydroxyl and methoxyl groups) or dimethylchromene (formed by cyclization between vicinal hydroxyl and monoprenyl groups) (44). Those isoflavonoids that are dietary phytoestrogens generally fall into the class of simple isoflavones and their glycosides although coumestrol (Figure 2*A*), a more complex isoflavonoid derivative, is also a


Oxidase
Dirigent protein
Coniferyl alcohol
(–) Pinoresinol
PLR NADPH
(–) Lariciresinol
SDH
(–) Matairesinol
PLR NADPH
(+) Secoisolariciresinol
Enterolactone
UDPG
(+) Secoisolariciresinol diglucoside
Enterodiol

phytoestrogen. The number of known isoflavone glycosides [e.g., genistin (genistein 7-*O*-β-D-glucopyranoside)], is small when compared with the vast range of known flavonoid glycosides, and *O*-glycosides predominate.

Natural sources from which isoflavonoids and their glycosides have been isolated are listed in an excellent review by Dewick (44). However, relatively few species provide dietary isoflavones to humans. These include soybean (the seeds of which accumulate high levels—milligram amounts—of daidzein and genistein and their glycosides plus smaller amounts of free and conjugated glycitein and coumestrol), chickpea (biochanin A), and alfalfa sprouts (formononetin glycosides and coumestrol) (58, 156, 190). Figure 2 shows the structures of these compounds, along with that of the human estrogen 17β-estradiol. Note the similarity in structure between estradiol and isoflavone (Figure 2*A*), with the distance between the 7- and 4′-hydroxyl groups on isoflavones almost identical to that between the C3 and C17 hydroxyls of estradiol. *O*-methylation of isoflavones decreases their estrogenicity as determined by in vitro ER-binding assay, with formononetin and biochanin A less potent than daidzein and genistein, respectively. Isoprenylation of methylated isoflavones may increase estrogenic activity (160), although prenylated isoflavonoids are not common or quantitatively important components of the human diet.

Coumestrol has higher binding affinity for ER than has genistein, and exhibits strong estrogenic activity, similar to that of estradiol, in the rat uterotrophic assay (180). Coumestrol can suppress estrous cycles when fed to female rats, and negatively affects the sexual behavior of male offspring (193), as well as having potential mutagenic effects (50). Coumestrol also has metabolic effects that are independent of its estrogenicity, including increasing lipid synthesis and glycogen catabolism in perfused rat liver (145). Because the main sources of coumestrol are alfalfa and clover, these potentially deleterious effects are probably more relevant for veterinary than for human medicine, although clover is used as a medicinal plant (143).

One gram of powdered soybean chips contains nearly 800 μg of daidzein and over 500 μg of genistein (primarily as glycosides), but one gram of soy protein still retains high levels of approximately 150 μg of daidzein and 250 μg of genistein. Highly processed soy products such as miso and soy sauce contain much lower to negligible levels of genistein than does tofu, a major source of isoflavones in the Asian diet. In humans eating a soy-rich diet, ingested isoflavone levels can be very high, as determined by urinary excretion (108).

Figure 3 Biosynthesis and metabolic conversions of the major dietary lignans. The reactions in gray shading represent microbial metabolism of plant lignans to the "mammalian lignans." The enzymes are PLR, pinoresinol/lariciresinol reductase and SDH, secoisolariciresinol dehydrogenase. Glucosyltransferase(s) and β-glucosidases catalyze the interconversions between secoisolariciriesinol and its diglucoside. UDPG, uridine diphosphate glucose.

A full understanding of dietary levels of phytoestrogens is essential for epidemiological studies in which phytoestrogen intake is usually estimated from food frequency questionnaires. Therefore, much effort has gone into developing methods for the accurate quantification of phytoestrogens in plants, plant extracts, and botanicals, as well as in body tissues and fluids. The methods range from those that target specific compounds, such as high-performance liquid chromatography (HPLC) coupled to UV spectroscopy and/or mass spectrometry (17, 96) and immunological methods such as enzyme-linked immunosorbent assay (19), to methods that do not chemically identify the active compound(s), such as in vitro estrogencity tests (the rodent uterotrophic assay) (47), microtitration assays with human ER (65), or analysis of ER binding using a reporter gene assay in yeast (134). Generally speaking, HPLC with UV detection is one of the simplest methods for determination of isoflavone phytoestrogen levels, both in plant extracts and animal tissues or body fluids (119, 189). However, the sensitivity of this method has been questioned, and isotope dilution gas chromatography mass spectrometry with selected ion monitoring was developed as a significantly improved method for accurate quantitation (129). The method involves inclusion of deuterated internal standards at the initial extraction stage, and has resulted in an expanded view of those plant sources that contain isoflavones, albeit at low levels (129) (see Table 1).

Several databases and compendia have been developed to complement food frequency questionnaires, based on quantitative analysis of isoflavones, coumestans, and lignans by the methods outlined above (78, 150, 156). In one such study, these compounds were determined in each of 112 food items/groups. As expected, high levels of genistein and daidzein, as well as substantial amounts of coumestrol, were found in traditional soy-based foods, as well as soy protein isolate, soy concentrate, or soy flour added to foods. Various types of sprouts and dried fruits, garbanzo beans, asparagus, garlic, and licorice, were also substantial contributors of dietary phytoestrogens (78, 150). Table 1 provides a summary of isoflavonoid phytoestrogen levels in some of the food sources analyzed by Adlercreutz and coworkers (129). All the species reported to contain significant levels of isoflavones are members of the Leguminosae.

The placenta does not appear to be a barrier to daidzein and related phytoestrogens (41), so mothers with high dietary isoflavone intake could provide the fetus with significant exposure to the compounds. There has also been considerable interest and concern about the effects of soy phytoestrogens in infant formula. The concern is based on results of some animal studies which, for example, have shown that neonatal exposure of mice to 50 mg genistein/kg per day for five days resulted in a level of uterine adenocarcinoma at 18 months of age similar to that induced by diethylstilbestrol (144). However, infant soy milk formulas do not appear to have estrogenic effects in the reproductive tracts of mature mice (152), and a study of four soy infant formulas revealed levels of genistein and daidzein resulting in a daily dose rate of total isoflavones of approximately 3 mg/kg body weight (84), much lower than that inducing cancer in neonatal mice.

TABLE 1 Levels of isoflavones and lignans in various food sources. Values (in nanomoles per gram dry weight) are taken from Reference 129 and were determined by isotope dilution gas chromatography mass spectrometry with selected ion monitoring

Plant species (common name)	Genistein	Daidzein	Secoisolariciresinol	Matairesinol
Soybean	993–3115	413–2205	<1–8	<1
Kidney bean	<1–19	<1–2	2–4	<1
American groundnut	4–30	<1	<1–2	<1
Chickpea	3–8	<1–8	<1	0
Pea	<1	<1	<1	<1
Lentil	<1	<1	<1	<1
Kudzu root	467	7283	<1	<1
Flaxseed	0	0	10,247	30
Sesame seed	<1	6	2	17
Sunflower seed	<1	<1	17	0
Peanut	2	1	8	<1
Wheat bran	<1	<1	3	0
Barley (whole grain)	<1	<1	2	0
Rye bran	0	0	4	5
Strawberry	0	0	33	<1
Cranberry	0	0	29	0
Blueberry	0	0	23	0
Raspberry	0	0	4	0
Red cabbage	<1	<1	4	<1
Broccoli	<1	<1	11	<1
Garlic	0	0	11	<1
Zucchini	0	0	23	<1
Carrot	0	0	10	<1
Beetroot	0	0	3	<1
Black tea	Trace	Trace	73	12
Green tea	Trace	Trace	75	5

Dietary supplements containing isoflavone phytoestrogens are now widely available from health food stores and through the Internet. They are taken for various reasons, ranging from a belief in their anticancer potential, ability to alleviate a range of postmenopausal problems ("natural" hormone replacement therapy), and even breast enhancement. Major active compounds in these formulations are the soy phytoestrogens, the isoflavonoid puerarin from Kudzu vine (*Pueraria lobata*),

and formononetin from clovers and fenugreek (18). Several plant species that are used as herbal medicines also contain isoflavonoids with estrogenic activity. Clover has already been mentioned in this respect. Another example is the root of licorice (*Glychirriza echinata*), which contains an estrogenic isoflavan (glabridin) and isoflavene (glabrene) (176).

Black cohosh (*Actaea racemosa* syn. *Cimicifuga racemosa*) is a North American perennial plant that is being developed as a natural alternative to hormone replacement therapy for treating menopausal symptoms. The active principle in black cohosh was thought to be formononetin, the compound responsible for the estrogenic effects of clover. However, this was discounted in recent studies that failed to detect this compound in a number of populations of the plant from throughout the United States, and in several commercial preparations (94).

The levels of phytoestrogens in soybean seed can vary significantly depending on cultivar and environmental factors (54). More than 75% of the soybeans currently grown in the United States are transgenic for resistance to the herbicide glyphosate (Roundup^{TM}). Glyphosphate inhibits biosynthesis of shikimate, a precursor of the amino acid phenylalanine from which isoflavones are derived. It was recently shown that application of Roundup^{TM} at various doses in the field does not appear to affect phytoestrogen levels in soybean (51).

Biosynthesis

Genistein is biosynthetically the simplest isoflavonoid. It is a central intermediate in the formation of more complex isoflavonoids with roles in establishing or inhibiting interactions between plants and microbes (48). Isoflavonoids are formed by a branch of the flavonoid biosynthetic pathway, and originate from a central flavanone intermediate [naringenin (4′,5,7-trihydroxyflavanone) in genistein biosynthesis, liquiritigenin (4′,7-dihydroxyflavanone) in daidzein biosynthesis] that is ubiquitously present in plants. For entry into the isoflavonoid pathway, the flavanone first undergoes abstraction of a hydrogen radical at C-3 followed by B-ring migration from C-2 to C-3 and subsequent hydroxylation of the resulting C-2 radical. This reaction requires reduced nicotinamide adennine dinucleotide phosphate (NADPH) and molecular oxygen, and is catalyzed by a microsomal cytochrome P450 enzyme [2-hydroxyisoflavanone synthase (2-HIS), loosely termed isoflavone synthase (IFS)] (Figure 2*B*). The IFS reaction is stereoselective, and (2*R*)-flavanones are not substrates. The resulting 2-hydroxyisoflavanone is unstable and readily undergoes dehydration to yield genistein or daidzein at acidic pH. It has been suggested that a specific dehydratase enzyme catalyzes the 2-hydroxyisoflavanone to isoflavone conversion in planta, and such an enzyme has been purified from *Pueraria lobata*, although it has not been characterized at the molecular level and is not necessary for production of isoflavones in transgenic plants expressing IFS alone (92, 119).

Because of the lability and low abundance of IFS, the enzyme eluded molecular characterization for many years. However, cDNAs encoding IFS have now been cloned from soybean, licorice, and other species (8, 92, 170), largely aided by data

mining of expressed sequence tag (EST) libraries. The enzyme converts liquiritigenin or naringenin to the corresponding 2-hydroxyisoflavanone in the presence of NADPH. It will be interesting to analyze the reported nonlegume sources of isoflavones listed in Table 1 for the presence of orthologs of legume IFS. If verified, this would significantly expand the number of known species in which IFS enzymes have evolved.

Daidzein differs from genistein by lack of a hydroxyl group at the 5-position. This hydroxyl group arises naturally from the head-to-tail condensations of malonyl CoA residues during the formation of naringenin chalcone by chalcone synthase. Loss of the 5-hydroxyl occurs during formation of the polyketide intermediate that cyclizes to give the chalcone, and is catalyzed by a specific NADPH-dependent reductase incorrectly named "chalcone reductase" (Figure 2*B*). Chalcone reductase is not present in all plants. The presence of both daidzein and genistein in soybean seeds and some clovers suggests that a significant proportion of the polyketide intermediate can escape the action of chalcone reductase. In contrast, legumes such as alfalfa produce predominantly 5-deoxy-isoflavonoids such as formononetin.

Formononetin from alfalfa and clovers, and biochanin A from chickpea, are methylated on the 4′-position of the B-ring. The biosynthetic origin of this 4′-methyl group has been a topic of controversy for many years, since it was observed that the only *O*-methyltransferase activity that could be detected in cell-free extracts from plant cultures producing formononetin catalyzed methylation at the A-ring 7- rather than the B-ring 4′-position of daidzein or genistein (53, 71) (Figure 2*B*). Radiolabeling studies in alfalfa cell suspension cultures provided convincing evidence to indicate that daidzein is not an intermediate in the biosynthesis of formononetin (120). This observation was made many years earlier in studies on the biosynthesis of phytoalexins in elicitor-treated alfalfa seedlings (45), and it was then proposed that *O*-methylation was an integral component of the aryl migration reaction catalyzed by IFS (45). This idea lost support with the later demonstration that the IFS reaction occurs with no requirement for B-ring methylation (100). Two different explanations for the origin of the B-ring methoxyl group have now been proposed. In both, the substrate for *O*-methylation is the 2-hydroxyisoflavanone product of the IFS reaction formed prior to dehydration to yield the isoflavone (Figure 2*B*). In one model, based on studies of the subcellular localization of the previously characterized alfalfa isoflavone 7-*O*-methyltransferase (IOMT) (120) and its reaction mechanism as determined from its 3-dimensional crystal structure (205), the IOMT, when physically associated in a complex with IFS, catalyzes 4′-*O*-methylation of one specific steroisomer of the 2-hydroxyisoflavanone intermediate (205). This model is supported by the observation that IOMT, which is an operationally soluble enzyme in healthy, unchallenged alfalfa cells, relocates to the endoplasmic reticulum following treatments that induce the membrane-anchored IFS (120). In a second model, based on studies with elicitor-treated licorice cell cultures, a separate and distinct enzyme from IOMT, with no daidzein 7-*O*-methyltransferase activity, catalyzes the 4′-*O*-methylation of 2-hydroxyisoflavanone (9). This latter *O*-methyltransferase has strong sequence

homology to a pterocarpan A-ring *O*-methyltransferase implicated in the biosynthesis of the isoflavonoid phytoalexin pisatin in pea (153).

Conversion of isoflavones to their glucose and glucose-malonate conjugates is catalyzed by glycosyl transferase and malonyl transferase enzymes that have been characterized enzymatically, particularly from chickpea (101, 102), but not yet at the molecular level. It is likely that some plant glycosyltransferases involved in conjugating compounds with phenolic hydroxyl groups may have relatively broad substrate specificity (186). For example, a glycosyltransferase from *Eucalyptus perriniana*, a species that has not, to the author's knowledge, been reported to produce isoflavonoids, efficiently converts daidzein to daidzin (daidzein 7-*O*-glucoside) (142). Likewise, enzymes in *Arabidopsis thaliana*, presumably involved in glycosylation of endogenous flavonols, can glycosylate genistein when this "foreign" compound is produced transgenically (119).

Coumestrol, a member of the coumestan class of isoflavonoids (Figure 2*A*), is derived from daidzein by a series of reactions that include hydroxylation/oxidation at C2 and ring closure with loss of water between the hydroxyl on C4 of the heterocycle (isoflavone numbering) and a hydroxyl on C2′ of the B-ring (48). Although this pathway was proven by radiolabelled precursor feeding studies, the enzymes that catalyze the late stages of coumestan biosynthesis have yet to be characterized at the molecular level.

Bioavailability and Metabolism

As indicated above, most flavonoids and isoflavonoids exist in the plant as glycosidic conjugates, generally located in the central vacuoles of the cells. Bioavailability of these true dietary components depends on relative uptake rates of conjugated and free forms; hydrolysis of glycosides by gut bacteria or gut wall enzymes; further metabolism, for example to glucuronides within the liver followed by enterohepatic circulation; and excretion rate into urine and bile.

The malonyl glucosides of daidzein and genistein found in soybean are labile and readily degraded to the nonacylated glucosides following cooking. The free aglycones, but not the glycosides, are absorbed from rat stomach. However, once in the small intestine, brush border lactase phlorizin hydrolase can effectively hydrolyze isoflavone glucosides (38). In humans, isoflavones appear in blood plasma at a more rapid rate and at higher levels following oral administration of the aglycones compared with the glycosides, and genistein and daidzein, but not their glycosides, are readily transported across human intestinal epithelial cell monolayers (171). Genistein attains higher plasma concentrations than daidzein when administered at the same level (164). This is because daidzein becomes more widely distributed within the body. However, genistein has greater bioavailability than daidzein, and overall bioavailability may increase if the compounds are ingested as their glycosides (164). This is important because many chemical intervention trials and in vitro studies in animal systems have utilized the free aglycones rather than the naturally occurring glycosides.

Although isoflavone ingestion results in increased levels of the parent isoflavone in plasma and urine, the compounds are also further metabolized. Although soy isoflavones exhibit estrogenic activity, their metabolite equol (Figure 2*A*), formed from daidzein by intestinal bacterial activity, is significantly more estrogenic and may be largely responsible for the physiological effects of isoflavone intake (165). Equol and other isoflavone metabolites can also be delivered into the human diet from cow's milk (129). The levels of urinary equol in humans eating a soy-rich diet can be approximately 100-fold higher than those observed in adults who consume little soy products in their diet. Equol binds to both human ER forms (ERα and ERβ), has high antioxidant activity, and is relatively stable. Conversion of soy isoflavones to equol can be influenced by factors in the diet affecting microbial populations, such as carbohydrate and dietary fiber levels (108, 168), a fact that can complicate epidemiological and dietary intervention studies. There is now evidence to suggest that the ability to produce equol is not the same in all humans, and that some individuals do not have this ability, presumably because they are host to a different population of intestinal microorganisms (165). In several studies, excretion of equol only occurred in approximately 35% of cases, whether male or female (168).

Biochanin A and formononetin are rapidly demethylated following ingestion, giving rise to genistein and daidzein, respectively. In addition to metabolism by gut bacteria, the demethylation reactions can also be catalyzed by a range of different cytochrome P450 enzymes in the liver, with some catabolizing biochanin A but not formononetin, and vice versa (181). The demethylated compounds can be further hydroxylated by hepatic enzymes (181).

Soy isoflavones are also converted to a range of conjugates by mammalian metabolism. Following chronic dosing of daidzein, its 7-glucuronide (54%), 4′-glucuronide (25%), monosulfate (13%), free aglycone (7%), sulfoglucuronide (0.9%), diglucuronide (0.4%), and disulfate (<0.1%) could be detected in human urine using liquid chromatography electrospray ionization tandem mass spectrometry (29). *O*-desmethlyangolensin (Figure 2*A*) is also a metabolite of daidzein in humans, and a fluoroimmunoassay was recently developed for this compound (117). The glucuronides retain weak estrogenicity and may be able to activate natural killer cells that help combat cancer at physiological concentrations (203). Similar metabolites of genistein to those listed above are found in rat and man. These include genistein glucuronide, dihydrogenistein glucuronide, genistein sulphate, dihydrogenistein, and 6′-hydroxy-*O*-desmethylangolensin (31). However, rat liver slices or isolated hepatocytes only catalyzed the glucuronidation of genistein (31).

Potential Health Beneficial Effects of Isoflavonoid Phytoestrogens

HORMONE-DEPENDENT CANCERS The structural features of genistein and daidzein confer ability to bind ER and sex hormone binding proteins, and isoflavone

phytoestrogens can exert both estrogenic and antiestrogenic activity, the latter by competing with estradiol for ER binding. Genistein and equol displace bound estrogen and testosterone from human sex hormone binding globulin (SHBG), and can affect the cellular levels of SHBG (151). Thus, genistein and other phytoestrogens could potentially affect clearance rates of androgens and estrogens and therefore the availability of the hormones to target cells. Genistein and coumestrol exhibit significantly higher binding activities for ERβ than for ERα (129); ERβ appears to be the prominent ER form in prostate secretory epithelium, brain, urinary tract, and possibly also breast cells. This may be an important factor for the apparent links between isoflavonoid phytoestrogen intake and reduced risk of hormone-dependent cancers (129).

Significant correlations exist between an isoflavone-rich soy-based diet, urinary isoflavone levels, and reduced incidence of breast cancer or mortality from prostate cancer in humans (35, 64, 159). An early epidemiological study of Singapore Chinese women that included 420 healthy controls and 200 with histologically confirmed breast cancer indicated that soy consumption was directly correlated with reduced risk of cancer (109). The positive effects of a soy diet appeared to be dietary rather than genetic because Asians who immigrate to the United States and adopt a Western diet are at higher risk of breast and prostate cancers. Similar observations have been reproduced in many, but not all, subsequent studies undertaken up to the present day, and the effects may also be in part attributed to high dietary lignan levels (35). Based on knowledge of diet and urinary excretion levels of daidzein, genistein, and equol in Japanese compared with American or European subjects, the isoflavonoids found in soy products were proposed to be the agents responsible for reduced cancer risk. However, cancer risks are also low in some Asian populations that do not have a high soy diet (64), and a study with postmenopausal Dutch women failed to reveal a link between dietary isoflavone levels and breast cancer risk (42).

Neonatal administration of genistein effectively protects against chemically induced mammary tumors in rats (59). The protective effects include increased latency, reduced incidence and multiplicity of tumors, and more rapid maturation of undifferentiated end buds to differentiated lobules (33). These effects, which appear to be ER mediated, are associated with increased epidermal growth factor receptor (EGFR) and progesterone receptor (PR) expression in the prepubertal rat mammary gland (33). Thus, genistein may induce early mammary gland differentiation resulting in a less active EGF signaling pathway in adulthood that, in turn, suppresses development of mammary cancer (106). Although the author is aware of no reported clinical trials documenting effects of controlled dietary supplementation with genistein on breast cancer incidence in humans, a high soy diet containing up to 45 mg of isoflavones per day can cause changes in the menstrual cycle that may help reduce cancer risk. In contrast to the results of these studies, dietary feeding of supraphysiological concentrations of daidzein to female rats neither caused significant toxicity to the reproductive tract nor provided protection against chemically induced mammary cancer (107). In studies in which rats

were fed a standardized soy extract instead of pure isoflavone, chemically induced mammary adenocarcinomas took longer to develop than in control animals, but at the end of the study no difference in tumor multiplicity or incidence was observed between treatment and controls (62).

Isoflavonoid phytoestrogens show complex effects on the growth of breast cancer cells grown in vitro. Biochanin A has a multiphasic activity on human mammary carcinoma cells, being stimulatory to growth at very low concentrations (less than 10 μg/ml), cytostatic at intermediate concentrations (40 μg/ml), and cytotoxic at higher concentrations (81). These effects appear to be ER dependent, and ER mRNA levels paralleled the growth rates of the cells at the different biochanin A concentrations (80). In a separate study, differential effects of genistein and coumestrol were observed in relation to ER and PR levels in the same mammary carcinoma cell line, with coumestrol behaving as an ER agonist and genistein showing features of a selective ER modulator (46). This latter activity might involve isoflavone-mediated selective recruitment of coregulatory proteins to ERβ to trigger transcriptional pathways (13).

Biphasic growth stimulation (measured as DNA synthesis) followed by inhibition of human mammary carcinoma cells was also observed as a function of increasing concentrations of a number of other (iso)flavonoid compounds, including coumestrol, genistein, apigenin, luteolin, kaempferol, and the lignan enterolactone (see below) (188). This may be a concern, particularly for women who already have initiated breast cancer, in view of low but significant phytoestrogen concentrations in some diets (188). One report suggests that low concentrations of genistein may antagonize the effects of the structurally related compound tamoxifen (Figure 2A) (40), a drug used as a chemopreventive for women at high risk for breast cancer. However, at high concentrations, such as those reached with a soy-rich diet, genistein is a strong cytotoxic agent against breast cancer cells with a mechanism independent of ER (123). Dietary intake levels might be the key to isoflavone phytoestrogens' mode of action and the balance between risk of, or chemoprevention from, breast cancer (123).

Genistein is unique among a number of flavonoid and isoflavonoid compounds tested in having both strong estrogen agonist activity and strong growth inhibitory activity against breast cancer cells (202). In contrast, equol has strong estrogen agonist activity but little growth inhibitory activity. Genistein is more effective in inhibiting growth of non-neoplastic human mammary cell lines than it is in inhibiting growth of mammary cancer cells (167). This supports the notion that early exposure to genistein may be important for breast cancer chemoprevention.

Prostate cancer is the second most frequent cause of cancer-related deaths in men in the United States, and there is no effective therapy for the disease once it becomes metastatic. Inverse relationships have been observed between high phytoestrogen intake and incidence of and mortality from prostate cancer (7), and prostate cancer cell growth in vitro and in vivo, and levels of androgen-related prostate-specific antigen, are all decreased by administration of genistein (37, 132). High concentrations of genistein do not appear to be toxic to the rat prostate

(60), although they may induce inflammation (prostatitis) (105). In a case control study of 83 prostate cancer cases and 107 controls carried out in the United States between 1996 and 1998, dietary intake of coumestrol and daidzein appeared to be more significantly related to reduced prostate cancer risk than did dietary intake of genistein (173). In the same study, there appeared to be a positive relationship between intake of the phytosterols campestrol and stigmasterol and prostate cancer (173). Daidzein only exhibited weak inhibitory effects on growth of benign and malignant human prostate epithelial cells, but its metabolite equol had potent inhibitory effects at micromolar concentrations (72). Thus, conversion of daidzein to equol may be an important factor in dietary prevention of prostate cancer.

OTHER CANCERS In addition to effects on breast and prostate cancers, genistein and related isoflavones also inhibit cell growth and/or development of chemically induced cancers in stomach, bladder, lung, and blood. Inhibition of the growth of human stomach cancer cell lines in vitro by genistein and biochanin A involves stimulation of a signal transduction pathway leading to apoptosis (198). When these cancer cells were transplanted into mice, biochanin A, but not genistein, significantly inhibited tumor growth. Genistein strongly inhibits growth of leukemia cells when targeted to them by linkage to a monoclonal antibody (183), and a prenyl isoflavone derivative (ipriflavone) was developed as an oral treatment for acute leukemias (147). The prenyl group might help target the isoflavone to hydrophobic sites of action.

A preliminary study, based on a multiethnic population-based case-control analysis of thyroid cancer conducted in the San Francisco Bay area, led to the conclusion that there may be a link between an isoflavone-rich diet and reduced risk of thyroid cancer (79). The study involved over 800 white and Asian women, both pre- and postmenopausal. However, the authors were cautious in interpreting the data, and indicated that further trials would be necessary before firm conclusions could be made.

There have been claims for a protective effect of isoflavones on colon cancer, but these are somewhat conflicting (3). At low concentrations, genistein induces the phase II detoxifying enzyme quinone reductase in colonic cells; biochanin A and coumestrol also have this ability, albeit less effectively, but daidzein and formononetin are inactive (192). Induction of a carcinogen detoxifying system could provide a partial explanation for anticancer effects of phytoestrogens.

In spite of the large number of studies supporting cancer chemoprevention by genistein, some studies have suggested a potential for opposite effects. These include increased numbers of carcinogen-induced aberrant crypt foci in the colons of rats fed genistein (66) and induced structural chromosome aberrations in human peripheral lymphocytes (104).

POSTMENOPAUSAL AILMENTS Estrogen deficiency in postmenopausal women can lead to overall bone loss resulting from increased bone resorption and decreased bone formation. This condition, referred to as osteoporosis, is a major public health

problem. There has therefore been considerable interest in the reports suggesting that soy isoflavones can attenuate bone loss associated with estrogen deficiency. Isoflavone phytoestrogens stimulate osteoblastic bone formation, inhibit osteoclastic bone resorption (52), and prevent overall bone loss in ovariectomized rats or mice (55, 85). Suggested mechanisms for these effects include stimulation of proliferation of osteoblast (bone forming) cells and protection of such cells from oxidative damage, and increased apoptosis of osteoclast (bone destroying) progenitor cells (112, 154, 196).

The above effects in animal model systems were confirmed in several controlled clinical trials with postmenopausal women, which indicated that an isoflavone-rich soy diet may attenuate bone loss in the lumbar spine and hip (12, 131, 140), and that this effect is due to isoflavones rather than to soy protein (12). However, such effects were not observed in premenopausal women (131). A recent study reported that habitual tea drinking may also increase bone mineral density in the lumbar spine and hip (194). It is possible that strong flavonoid antioxidants such as epicatechin gallate found in tea are responsible for this effect. Isoflavones also possess antioxidant properties, and there may therefore be more than one mechanism whereby plant natural products protect against osteoporosis.

Phytoestrogens may be effective in reducing other symptoms of estrogen depletion in postmenopausal women, such as hot flushes, night sweats, and vaginal dryness (11, 16). A recent study carried out in Spain on 190 postmenopausal women reported statistically significant improvements in hot flashes, with no negative side effects, in response to dietary supplementation with soy isoflavones administered through commercially available capsules (10). Red clover extracts are popular dietary supplements, with many perceived beneficial effects including reduction of postmenopausal symptoms (143). However, some studies with clover and soy have failed to confirm efficacy (16, 61). In one study, the group of women taking the dietary soy supplement reported increased incidence of insomnia (16). A recent review of 74 studies addressing effects of phytoestrogens on postmenopausal ailments concluded that, although these compounds may indeed have some degree of efficacy, this falls well short of that obtained from traditional hormone replacement therapy (68).

The potential beneficial effects of soy isoflavones on breast cancer risk have also been questioned in studies that specifically target postmenopausal women. For example, a study that included 88 breast cancer cases and 268 controls, selected from a large group of postmenopausal women participating in a breast cancer screening program in the Netherlands, failed to demonstrate a significant correlation between phytoestrogen intake (assessed as urinary phytoestrogen level) and cancer risk (182).

CARDIOVASCULAR DISEASE Results of epidemiological studies suggest that high dietary intake of isoflavones and/or flavonols may contribute to a low incidence of heart disease in Japanese women. Effects of isoflavones on cardiovascular health

may result from inhibition of low-density lipoprotein (LDL) oxidation (178, 179), an effect that may be enhanced by food sources rich in vitamim C (82); inhibition of proliferation of aortic smooth muscle cells, as concluded from a study with stroke-prone spontaneously hypertensive rats (148); and maintenance of the physical properties of arterial walls (185).

Postmenopausal women have increased risk of cardiovascular disease due in part to elevated cholesterol levels accompanying the loss of endogenous estrogen secretion. In premenopausal women, dietary genistein appears to improve plasma lipids (resulting in lowered LDL cholesterol), the ratio of total cholesterol to high-density lipoprotein (HDL) cholesterol, and the ratio of LDL to HDL cholesterol (133). Positive effects on cardiovascular risk profile were also concluded from a study of more than 900 postmenopausal women (99), although such effects were not observed in a separate study with a smaller sample size (43). The protective effects of soy phytoestrogens appear most significant in individuals with initially elevated cholesterol levels; phytoestrogen intake may have less or no effect on individuals with normal cholesterol levels (76, 178). On the basis of such findings, the U.S. Food and Drug Administration has approved use of food health claims for intakes of 25 g isoflavone-rich soy protein per day for the reduction of blood cholesterol levels (88). This would equate to approximately 6 mg of genistein per day. In a study on potential toxicology of phytoestrogens, the no-observed-adverse-effect level for genistein was estimated to be 120 mg/kg per day in rats (146). In rats, the hypocholesterolemic effect of a soy diet may involve interactions between the isoflavones and soy protein (149), whereas in cholesterol fed rabbits, attenuation of atherosclerosis by isoflavones does not require the presence of soy protein.

A comparison of the progression of atherosclerosis in ovariectomized cynomolgus monkeys treated with equine estrogen or soy isoflavones indicated that the plant products, although providing protection, were less effective than mammalian estrogen (30). Finally, soy intake may have beneficial effects with respect to obesity (with links to cardiovascular disease) and diabetes, although it is not clear whether this is due to isoflavones or other components (20).

COGNITIVE FUNCTION Depletion of estrogen at menopause may be associated with increased risk of neurodegenerative diseases, and estrogen replacement therapy improves episodic and semantic memory in postmenopausal women. Remarkably, in one study a high soy diet improved memory within weeks in both young male and female human volunteers (57). A series of studies on brain structure, learning, memory, and anxiety in rats led to the conclusion that high consumption of dietary phytoestrogens over a relatively short time period can significantly alter the volume of sexually dimorphic brain regions, increase anxiety, and improve learning and visual-spatial memory in females but not in males (115). Generally, males outperform females in a maze test, but this sexual dimorphism was reversed when the animals were placed on a phytoestrogen-rich diet (122). When the male rats were "feminized" by treatment with the androgen receptor blocker flutamide,

they then responded to phytoestrogen in a similar manner to females (121). The effects of phytoestrogen on sexually dimorphic memory performance were not associated with changes in brain aromatase levels (115). These studies are exciting in view of the potential importance of estrogens in brain and neural disorders such as Alzheimer's disease, especially in women.

A recent study indicated that genistein has comparable ERβ-mediated antiapoptotic properties to 17β-estradiol in primary cortical neurons (118). Understanding more about how isoflavone phytoestrogens may affect cognitive function will be an exciting goal for the future.

REPRODUCTION AND FERTILITY The phytoestrogens were first identified by their effects on fertility in sheep grazing high-isoflavone containing clovers. Subsequently, it was suggested that decreasing fertility rates in some human populations may be a result of exposure to environmental estrogens. However, little experimental evidence supports this concept. Genistein and the flavanone phytoestrogen 8-prenylnaringenin (see below) actually increased the fertilizing ability of mouse sperm in vitro at submicromolar concentrations, apparently by an ER-independent mechanism (1). In male rats, long-term exposure to genistein in utero, during lactation, and for up 130 days post gestation had no apparent adverse effects on gametogenic function and sperm count (158). In human males, a two-month exposure to high dietary isoflavone levels likewise had no negative impact on semen function (137).

Elevation of plasma isoflavone levels resulting from inclusion of soy milk in the diet correlates with elevated SHBG levels in postmenopausal women (151). However, a randomized double-blind trial with 34 premenopausal women fed either 100 mg of isoflavones per day or placebo over a one year period indicated no alterations in menstrual behavior (127). A six-month intake of phytoestrogens had no effect on endometrial histology in a group of 19 postmenopausal women (16). However, soy isoflavones have been reported to reduce the incidence of menstrual-linked migraines that have been associated with fluctuations in estrogen and progestin levels (25).

On balance, the evidence suggests that isoflavone phytoestrogens may represent a safe and natural dietary supplement for alleviating many ailments that affect older women. However, significant physiological effects may result from exposure to isoflavone phytoestrogens during development of the female reproductive system, at least as demonstrated in animal models. Thus, neonatal exposure to genistein induces ERα expression and multioocyte follicles in the maturing mouse ovary (89), and use of DNA array analysis indicated that genistein causes similar changes in a specific set of gene transcripts to those caused by an estradiol derivative in developing rat uterus (141). Japanese infants at birth can exhibit high levels of isoflavone phytoestrogens passed through the placenta from the mother (6), and, although it has been suggested that this could be an important factor for reduced breast cancer risk later in life, there are also concerns about potential deleterious effects.

INFLAMMATION AND IMMUNITY Licorice root is commonly used in Chinese traditional medicine for treating inflammation, allergies, and asthma. Although licorice contains bioactive triterpenes and chalcones, the isoflavonoid licoricidin was recently shown to inhibit lyso-platelet activating factor (PAF) acetyltransferase, making it a strong candidate for the active antiallergenic principle (21). An isoflavone phytoestrogen–rich diet has also been shown to reduce inflammatory markers in a guinea pig model of asthma, although this was accompanied by potentially detrimental leakage of protein into the airspace of the lungs following challenge with an aerosol containing ovalbumin, to which the guinea pigs had been previously sensitized (155).

Additional concerns about the safety of infant exposure to soy-based formulas have been raised based on the results of experiments addressing effects on the thymus. Injection of genistein into ovariectomized adult mice at a concentration that produced similar serum genistein levels to those reported in soy-fed human infants resulted in significant loss of weight of the thymus gland, apoptosis of thymocytes leading to drastically reduced thymocyte numbers, changes in spleen cell numbers, and reduced humoral immunity (199). In a subsequent study, the same treatment reduced cellular immunity (200). In both studies, the effects of genistein were concluded to occur through ER-dependent and independent pathways. The implications of these studies for the development of the human immune system are not yet understood. Essentially opposite effects of genistein on spleen and thymus have been reported in studies with rats (70), and studies with adult humans have suggested that exposure to genistein might improve immune surveillance associated with increased levels of interleukin-6, at least in females (90). No significant effects were seen in these studies with males, and other inflammatory markers and cytokines were unaffected by genistein intake (90). Finally, genistein has been reported to increase host immunity in a mouse tumor model system, and this was proposed to involve increases in the activities of cytotoxic T cells and natural killer cells (69).

Metabolic Engineering of Isoflavone Phytoestrogens

As the entry point enzyme into isoflavonoid biosynthesis, IFS is the key step for engineering isoflavone production into nonleguminous plants that lack the pathway. Such a metabolic engineering strategy could serve two functions: to validate or invalidate the concept of health promotion by isoflavones by providing near isogenic material containing different concentrations of isoflavones in their naturally bioactive forms for animal dietary intervention trials, and if efficacy is validated, to provide new, value-added food crops for human health enhancement. To establish proof of principle for metabolic engineering of isoflavones, soybean IFS was introduced into *Arabidopsis thaliana*, corn, and tobacco (92, 119, 201). Free genistein does not accumulate but is in *Arabidopsis* converted to a series of glucose and rhamnose conjugates, including the rhamnoglucoside (119), reflecting the glycosylation pattern of the endogenous *Arabidopsis* leaf flavonols, kaempferol and quercetin.

The above transgenic lines only accumulate genistein conjugates to very low levels. This could be a result of the IFS activity level, substrate availability, substrate channeling, or product turnover. Various strategies have been taken to address the problem of substrate availability for engineered IFS. Upregulation of flavonoid synthesis in maize Black Mexican Sweet cell cultures expressing soybean IFS was achieved by expression of a chimeric transcription factor (CRC) containing the maize C1 and R transcription factor coding regions; this led to low levels of genistein production, from undetectable levels in the absence of CRC expression (201). Overexpression of chalcone isomerase in *Arabidopsis* expressing IFS led to a threefold increase in flavonol levels, but this was not accompanied by a corresponding increase in genistein conjugates. Likewise, genistein production in transgenic *Arabidopsis* was not increased in the *pap1-D* genetic background in which anthocyanin production is strongly upregulated (119). Thus, metabolic channeling through the endogenous pathways of flavonoid biosynthesis in *Arabidopsis* results in limitations to flux through the introduced IFS. This is explained by competition between flavanone 3-hydroxylase (F3H, the entry point into the flavonol pathway) and IFS for their common substrate naringenin. This idea is supported by the high-level production of genistein conjugates following expression of soybean IFS in the *tt3/tt6* mutant of *Arabidopsis*, which lacks F3H (119).

Although *Medicago* species such as alfalfa possess IFSs capable of producing genistein, this compound does not naturally accumulate in alfalfa. Rather, the 5-deoxy class of isoflavonoids is produced, due to the coexpression of IFS and chalcone reductase. These compounds, primarily formononetin and medicarpin conjugates, are produced constitutively in roots but not in leaves, but the corresponding aglycones accumulate in many tissues following microbial challenge. In contrast to the situation in *Arabidopsis*, ectopic expression of IFS under the CaMV 35S promoter leads to high levels of genistein accumulation in alfalfa leaves (B. Deavours & R.A. Dixon, unpublished results). This might result from the fact that the constitutively formed flavonoids in alfalfa are primarily flavones rather than flavonols (83), such that competition between IFS and F3H is not an issue.

Metabolic engineering studies for human health enhancement are in their infancy, and their relevance for the marketplace will be dictated by the emerging evidence for or against potential advantages and, of course, safety. In soybean, which is already marketed as a health-friendly food in view of its natural phytoestrogen content, genetic modification has been targeted toward obtaining more reproducible levels of daidzein and genistein, which vary depending on environmental conditions (77).

Phytoestrogens and Sex Determination in Plants

Information from plant EST and genomic sequencing projects to date would tend to suggest that plants do not contain functional orthologs of mammalian ER. Two low-abundance EST sequences from the model legume *Medicago trunctula* have homology to ERα and ERβ respectively, but similar sequences were not found

in other plant EST databases. The possibility that phytoestrogens might possess "estrogenic" functions within the plant is, therefore, perhaps fanciful. Nevertheless, preliminary data from the "osage orange" (*Maclura pomifera*, Moraceae) indicate that male and female branches of this dioecious species contain very different levels of a compound(s) able to activate human ER in yeast (124). *M. pomifera* fruit contain up to 6% dry mass of the di-prenylated isoflavone derivatives pomiferin and osajin, with smaller amounts of prenylated isoflavones and flavones with known in vitro ER activation activity in other parts of the plant (111). Whether the ER-activating compound(s) of *M. pomifera* truly play a role in sex determination in this species has not been determined.

FLAVONOID PHYTOESTROGENS

Occurrence

Although flavonoids occur ubiquitously in higher plants, their potential as phytoestrogens has received less attention than that of the isoflavonoids. Flavonoids typically either exhibit negligible or low estrogenic activity, particularly those found in plants that are a significant component of the human diet. Although weakly estrogenic flavonoids do occur in edible plants that are also used medicinally, such as mugwort (*Artemisia vulgaris*, used for inducing regular menstruation) (110), most interest in the flavonoids is associated with their antioxidant activity and potential roles in reducing heart disease (74, 157). Nevertheless, some flavonoids, particularly flavones, are better inhibitors of the aromatase cytochrome P450 that converts androgens to estrogens than are the isoflavones (93), and could therefore potentially affect estrogen levels in women. Limited reports suggest that commonly occurring flavonoids, such as the anthocyanidin pigments found in flowers and leaves (e.g., red cabbage) can exhibit ER-dependent responses in model systems (161), though they have very weak ER-binding activity. Because these compounds are often ingested in large quantities, their effects may warrant further investigation.

8-Prenylnaringenin (Figure 2*B*), a compound produced by hops (*Humulus lupulus*) and found in relatively small quantities [less than 20 μg/L, (177)] in some beers, is a much more potent flavonoid phytoestrogen. This molecule exhibits ER-mediated activity in mammalian cells at a concentration of approximately 1.0 μM (204), at least an order of magnitude lower than that of the parent flavonoid naringenin. Other prenyl-substituted flavonoids such as 6-prenylnaringenin, 6,8-diprenylnaringenin, and 8-geranylnaringenin also exhibit estrogenicity. The lipophilic substitution may help target 8-prenylnaringenin to ER or estrogen-metabolizing enzymes in vivo.

Along with a range of related prenylated chalcones and bitter acids, 8-Prenylnaringenin accumulates in the lupulin glands (peltate trichomes) found on the underside of the bracts of the female hop flowers used for flavoring beer. The compound when extracted from hops is a racemic mixture of both (+)- and (−)-enantiomers, both isomers showing similar binding characteristics to ER (22).

8-Prenylnaringenin is therefore most likely formed by nonenzymatic cyclization of prenylated naringenin chalcone, as the enzyme chalcone isomerase produces only the (−)-flavanone. Indeed, the prenylchalcone xanthohumol (a derivative of the prenylchalcone shown in Figure 2*B*, but with a methoxy group at position 6′ of the A-ring), which has little estrogenic activity (135), is present in the lupulin glands at much higher concentrations (two orders of magnitude) than the prenyl naringenins (22). The prenylation reaction uses dimethylallylpyrophosphate (DMAPP) as prenyl group donor. Direct enzymatic prenylation of naringenin can occur in plants, for example during biosynthesis of sophoroflavanone G (8-lavandulyl-2′-hydroxynaringenin) in *Sophora flavescens* (197). It is also possible that prenylation of naringenin, or conversion of prenylated chalcone to prenylnaringenin, can occur in beer during storage (75). Because of the increased bioactivity of prenylated flavonoids compared with the nonsubstituted compounds, flavonoid/chalcone prenyltransferases represent an important class of genes for metabolic engineering in plants.

Because of the differential solubility of prenyl naringenins and the hop bitter acids, spent hops (the plant material after extraction of the acids) represent an enriched source of phytoestrogens for herbal supplements or functional foods (22).

Resveratrol (*trans*-3,5,4′-trihydroxystilbene) (Figure 2*B*), a compound found in grapes (and therefore wine) and peanuts, is not strictly a flavonoid but is included here because it is biosynthetically related to the true flavonoids. The enzyme stilbene sythase (SS) is a polyketide synthase that is evolutionarily related to the chalcone synthase (CHS) of flavonoid biosynthesis. The same initial polyketide intermediate is formed by condensation of one molecule of 4-coumaroyl CoA and three molecules of malonyl CoA, but in SS the final cyclization is accompanied by a decarboxylation reaction. *SS* genes were cloned from a number of species, and are often phylogenetically indistinguishable from CHSs based on amino acid sequence alone (162). Structural features that distinguish CHSs from SSs have now been determined based on knowledge of the three-dimensional crystal structure of CHS (56) and rational site-directed mutagenesis experiments (91, 174).

Potential Health Beneficial Effects

8-Prenylnaringenin tests positive in a number of in vitro assays that assess potential health beneficial effects (172). For example, it inhibits the growth of human breast cancer cells in vitro, inhibits bone loss in ovariectomized mice (138), and inhibits the CYP1A2 cytochrome P450 involved in procarcinogen activation (73). It binds similarly to both ERα and ERβ, and its estrogenic activity in vitro is greater than that of genistein, daidzein, or coumestrol. However, tests in mice have shown that estrogenic effects of 8-prenylnaringenin supplied in drinking water require concentrations approximately 500-fold higher than those found in beer (135). Likewise, the levels of 8-prenylnaringenin present in products advertised for breast enhancement are insufficient to exhibit estrogenic activity at the level of the uterus

based on studies in mice (32). However, there has been some concern expressed about unrestricted intake of hop-based products with high phytoestrogen activity (135), and it has even been suggested that 8-prenylnaringenin might be responsible for menstrual disturbances in female hop workers (136).

Much of the interest in resveratrol centers on its perceived effects on cardiovascular health, and it has been postulated that its presence in red wine may be one factor in explaining the so-called "French paradox" (67), whereby a population with relatively high dietary fat intake has a lower than average incidence of cardiovascular disease. Resveratrol has also been reported to exhibit anticancer activity (87). It is a weak ER agonist/antagonist with equal binding activity for ERα and ERβ (24), but the relative extent of its in vivo estrogencity is cell type–dependent (67). Other activities of resveratrol consistent with phytoestrogenic properties and positive effects on cardiovascular health include enhancement of expression and activity of endothelial nitric oxide synthase (187), inhibition of platelet activation (49), and suppression of oxidative DNA damage (139). However, a study concluded that resveratrol is inactive in immature rat uterotrophic assays and questioned the degree of ER binding by resveratrol and its significance for the compound's cardiovascular activity (14).

LIGNAN PHYTOESTROGENS

Occurrence

Lignans are defined as dimeric phenylpropanoid (C_6-C_3) compounds, mostly linked 8-8′ (Figure 3), although many other linkage types are now encompassed within the term lignan. They are a common, structurally diverse class of plant natural product, and are widely distributed throughout the plant kingdom, where they function primarily in plant defense (116). Their major dietary sources are the outer layers of cereals and grains, with rye and flaxseed among the most important (Table 1). Analytical approaches for lignans include HPLC (103) and GC (129), with typical values of around 370 mg secoisolariciresinol per 100 g dry weight for flaxseed (2). Berries and garlic are also dietary sources of lignans (2) (Table 1).

Similar to isoflavones, the lignans exist in the plant as glycosides stored in the vacuole, and are converted to active phytoestrogens by microflora in the proximal colon. Thus, the glycosides of secoisolariciresinol and matairesinol are effectively converted to the so-called "mammalian lignans" enterodiol and enterolactone, respectively (Figure 3), and enterodiol can also be converted to enterolactone. It was recently shown that pinoresinol and lariciresinol (precursors of secioisolariciresinol), as well as syringaresinol, can also be metabolized to enterolactone (3). Thus, many lignans commonly found in whole grain products can be converted to phytoestrogens.

Serum enterolactone levels are elevated in men and women following provision of a diet rich in whole grains compared with refined grain foods (86). Consistent with the production of enterolactone by microbial metabolism of matairesinol

and secoisolariciresinol glycosides, taking oral antibiotics can significantly reduce levels of enterolactone (95).

Biosynthesis

The "monolignol" coniferyl alcohol, which is also a precursor of polymeric lignins, is the main building block of many lignans. Studies on the mechanism whereby two coniferyl alcohol molecules are linked to give the initial lignan moiety have revealed a unique biochemical mechanism for engendering stereospecific free radical coupling. Many lignans exist in a particular plant, or organ of that plant, in a single steroisomeric form. However, the dimerization reaction occurs through free radical coupling, initiated by single electron oxidation catalyzed by an oxidase such as a peroxidase or laccase (116). Such reactions, which also occur during lignin biosynthesis, are not stereochemically specific. The breakthrough in understanding lignan coupling came when Norman Lewis's group demonstrated the presence of a novel, noncatalytic protein present in the cell wall fraction of *Forsythia intermedia* stems. This so-called dirigent protein (Figure 3) was capable of determining the stereochemical course of the coupling of two molecules of coniferyl alcohol in the presence of an oxidase (36). In the absence of the dirigent protein the resulting lignan was a mixture of the two potential steroisomers, whereas the dirigent protein alone had no catalytic activity (36). Dirigent proteins are encoded by a large gene family with differential expression patterns in western red cedar, suggesting that there is considerable tissue and environmental specificity to the monolignol coupling reaction(s) (98).

The postcoupling steps in lignan biosynthesis vary greatly depending on the particular compound under consideration. In the flaxseed lignans, the major dietary sources of "mammalian lignans," the product of stereoselective monolignol coupling is (−)-pinoresinol (Figure 3). This is then converted to secoisolariciresinol via a two-step reduction. The enzyme involved, pinoresinol/lariciresinol reductase, is a member of a large gene family that includes isoflavone reductases from legumes (63). Finally, secoisolariciresinol is converted to the corresponding diglucoside (116). The availability of the genes encoding the enzymes of monlignol coupling and downstream reduction make genetic modification of lignan content a possibility.

Potential Health Beneficial Effects

As with the isoflavone phytoestrogens, evidence for health-promoting effects of lignans, particularly in the areas of hormone-dependent cancers and cardiovascular disease, has come from both epidemiological and chemical intervention studies (35, 79, 99), particularly in Scandinavia where the population consumes a diet rich in whole grain bread and grain fiber (5). In a review published in 1998, Adlercreutz concluded that breast cancer can be associated with low lignan levels in the United States, Finland, Sweden, and Australia (7). The epidemiological data on the relation between lignan consumption and prostate and colon cancers, as well

as coronary heart disease, were too limited to draw clear conclusions (7). In contrast, several studies on the effects of enterodiol and enterolactone on mammalian cancer cell lines in vitro have supported potential roles in cancer chemoprevention (191).

More recent studies have left a less clear picture of the link between lignan consumption and breast and prostate cancers. For example, a study with postmenopausal Dutch women (88 breast cancer cases and 268 controls), in which urinary enterolactone levels were determined over a nine-year period prior to developing breast cancer, failed to reveal a link between urinary mammalian lignan levels and breast cancer risk (42). Similarly, a large-scale study determining serum enterolactone levels in nearly 800 men who subsequently developed prostate cancer and over 2500 control men failed to support the hypothesis that high-circulating enterolactone levels protect against prostate cancer (169).

Evidence exists in humans that associations with reproductive risk factors for breast cancer differ according to cytochrome P450c17A (CYP17) genotype (130). In a recent study in which women in the highest tertile of dietary lignan intake had reduced breast cancer risk, the effect was more significant for premenopausal women with at least one CYP17A2 allele (130).

Dietary supplementation with 10% flaxseed to nude mice with established human breast tumor xenografts reduced tumor growth and metastasis (34). This was associated with decreased extracellular levels of vascular endothelial growth factor (VEGF), an important factor in angiogenesis and therefore cancer spread.

In a study in which hypercholesterolemic postmenopausal women that were put on a classical hormone replacement therapy were compared with those put on a flaxseed-rich diet, only the group on hormone replacement therapy had significantly improved cholesterol profiles and favorably modified markers related to cardiovascular health (113).

OTHER CLASSES OF PHYTOESTROGENS

Although not common dietary components, several other compounds with estrogenic activity are consumed by humans in herbal remedies. These include isoflavonoids and chalcone (isoliquiritigenin) from licorice root (discussed above). Rhubarb contains the phenylbutanone glucoside lindleyin, which binds to ERα and may be responsible for the biological effects of rhubarb extracts (184). Deoxymiroestrol is a potent phytoestrogen from the "rejuvenating" folk medicine Kwao Keur produced in Thailand from *Pueraria mirifica* (27). Ginseng contains several bioactive triterpenoid compounds, among which the glycoside ginsenoside stimulates proliferation of human breast cancer cell lines in an ER-dependent manner, and can activate ER element reporter gene constructs in transfected HeLa cells (26). The corresponding aglycone exhibited no significant activity. Extracts from *Polygonum*, *Cassia*, *Aloe*, and *Rheum* species enhance cell proliferation in estrogen-sensitive human breast cancer cell lines (128), and this is due to the

activity of anthraquinones. Emodin and 2,6-dihydroxyanthraquinone were among the most potent, and also inhibited 17β-estradiol binding to human ERα and ERβ (128).

Fresh corn products contain a mitogenic compound with estrogenic activity. This was discovered when corn cob bedding was shown to disrupt estrus cycling and sexual behavior in rats. The compound stimulates proliferation of ER-positive and ER-negative breast cancer cells, but does not compete for binding to ER (125). The activity copurifies with an isomeric mixture of linoleic acid derivatives with a tetrahydrofuran ring and two hydroxyl groups (THF-diols) that include 9, (12)-oxy-10,13-dihydroxystearic acid, and 10, (13)-oxy-9,12-dihydroxystearic acid, and these compounds disrupt estrus activity in rats at concentrations 200-fold lower than classical phytoestrogens (126). According to one report, fresh corn cobs and corn tortillas also contain these compounds, suggesting potential for human exposure (125).

Safflower (*Carthamus tinctorius* L.) seeds have long been clinically used in Korea to promote bone formation and prevent osteoporosis (97), and dietary supplementation with safflower seeds partially protected against ovariectomy-induced bone loss in rats (97). Preliminary studies indicated that this activity was associated with the polyphenolic fraction of the safflower seed, but the specific components responsible were not identified.

REASSESSING THE HEALTH BENEFICIAL EFFECTS OF PHYTOESTROGENS

Clearly, firm data unequivocally linking phytoestrogen intake to a reduced risk of disease are yet to be obtained for many diseases. This contrasts with the growing use of dietary supplements containing phytoestrogens. In two critical reviews (3, 4), Adlercreutz summarizes the status of the current body of work on phytoestrogens and human health. He concludes that there is a significant relationship between a high-isoflavone diet and reduced risk of breast cancer, but that this only holds if isoflavones are consumed throughout life, or at least before and during adolescence. There is also an inverse relationship between low plasma enterolactone levels and breast cancer. An isoflavone-rich diet may protect against prostate cancer, but more research is needed. There is no evidence to date indicating that isoflavones protect against colon cancer, or that high and continuous exposure to phytoestrogens may present an increased risk of breast cancer. Phytoestrogens have great potential for cardiovascular disease prevention, and may, in moderate amounts, help protect against several postmenopausal ailments. In all these areas, the activity of intestinal microflora is critical, as may be the parallel activity of other dietary components. Thus, a traditional Japanese diet not only includes high soy content, but is also low in fat. Therefore, chemopreventive effects of phytoestrogens suggested by epidemiological evidence may not translate to different races or cultures for which other dietary inputs differ. At present, no definite

recommendations can be made as to the dietary amounts of phytoestrogens needed for disease prevention.

One problem for the acceptance of potential health beneficial effects of phytoestrogens is that, to date, no specific mechanism(s) has been proven for their mode of action. The hormone-dependent cancers, atherosclerosis, and coronary heart disease are all associated in some way or other with sex hormones and their metabolism, and phytoestrogens clearly interfere with intracellular steroid hormone metabolism. This may be the key to their activity (3), but direct evidence is lacking. The other problem is that epidemiological studies yield essentially correlative data, and it is therefore difficult to distinguish between effects of phytoestrogens and other soy or grain fiber components, or indeed other lifestyle factors that might be associated with a particular type of diet.

A few recent studies on the effects of phytoestrogens on mammalian cells have utilized DNA arrays to obtain a more global picture of phytoestrogen-mediated changes in gene expression and to compare these to effects of mammalian estrogens such as estradiol (28, 39, 141). Unfortunately, the precision and resolving power of this technology is not matched by the overall experimental design. The problem is that application of pure chemicals to mammalian cells, either directly or through dietary supplementation, bypasses the important factors of dietary delivery and bioavailability, and dietary supplementation with food sources such as flaxseed or soy flour adds additional variables (i.e., all the other components of the supplemented food). It would be extremely helpful for assessing the true effects of dietary phytoestrogens in animal studies to be able to feed whole food sources that differ only in the phytoestrogen content. This can now be achieved for the isoflavones by genetic manipulation of the relevant pathways in transgenic plants (119, 201) such as *Arabidopsis*, rice, corn, and alfalfa, all suitable for animal feeding studies. It is expected that similar progress will soon be made with the lignans. Metabolic and transcriptional profiling of the plant tissue (175, 195) can be used to confirm that the dietary source of phytoestrogen(s) is not modified in other areas that could impact the experiments. Armed with such source material in which the phytoestrogens exist as natural glycosides in the correct cellular compartment within the plant, and in which their nature and levels can be precisely controlled, it should be possible to use transcriptome profiling in the animal to better understand the molecular impacts of a diet containing phytoestrogens on various target tissues. Such studies, in concert with more detailed epidemiological and chemical intervention work, may finally lead to the definition of "recommended daily doses" for phytoestrogens in relation to specific disease risks.

ACKNOWLEDGMENTS

The author thanks Cuc Ly for assistance with artwork. Work from the author's laboratory was supported by the Samuel Roberts Noble Foundation and the Oklahoma Center for the Advancement of Science and Technology Health Sciences Program.

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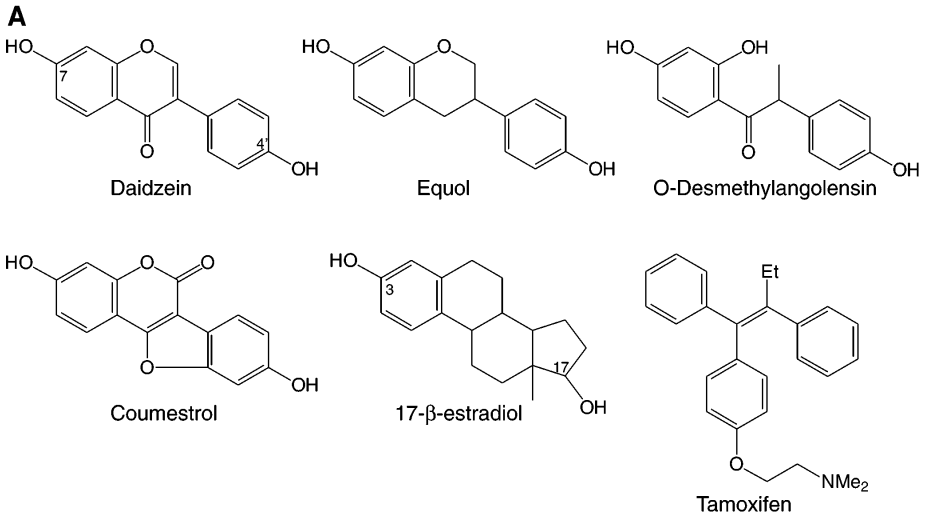

Figure 2 (*A*) Structures of isoflavonoid phytoestrogens and their metabolites, indicating structural similarities to 17β-estradiol. (*B*) The biosynthesis of isoflavonoid, flavonoid, and stilbene phytoestrogens. The enzymes are CHS, chalcone synthase; SS, stilbene synthase; CHR, chalcone reductase; PT, prenyltransferase; CHI, chalcone isomerase; IFS, isoflavone synthase (2-hydroxyisoflavanone synthase); IOMT, isoflavone *O*-methyltransferase; 2HIOMT, 2-hydroxyisoflavanone *O*-methyltransferase; and DH, 2-hydroxyisoflavanone dehydratase. The question mark indicates that the dehydratase is not essential because the reaction can occur nonenzymatically. The different classes of (iso)flavonoids are represented by different colors of shading: orange, stilbene; yellow, chalcone; pink, flavanone; blue, 2-hydroxyisoflavanone; green, isoflavone.

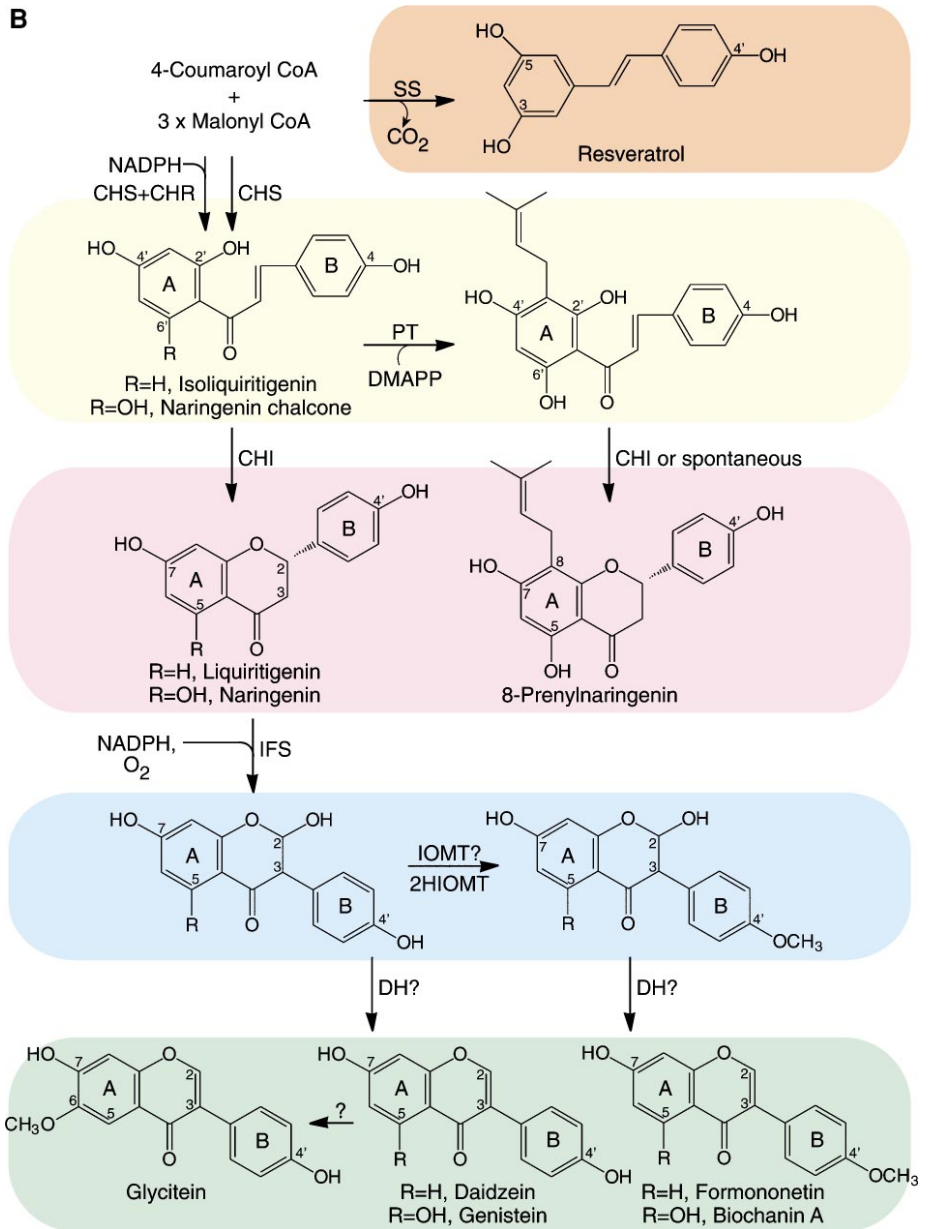

Figure 2 (*Continued*)