Microbial and Enzymatic Transformations of Flavonoids[⊥]

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Flavonoids are among the most ubiquitous phenolic compounds found in nature. These compounds have diverse physiological and pharmacological activities such as estrogenic, antitumor, antimicrobial, antiallergic, and anti-inflammatory effects. They are well-known antioxidants and metal ion-chelators. In the present review, biotransformations of numerous flavonoids catalyzed mainly by microbes and few plant enzymes are described in four different flavonoid classes, viz., chalcones, isoflavones, catechins, and flavones. Both phase I (oxidative) and phase II (conjugative) biotransformations representing a variety of reactions including condensation, cyclization, hydroxylation, dehydroxylation, alkylation, *O*-dealkylation, halogenation, dehydrogenation, double-bond reduction, carbonyl reduction, glycosylation, sulfation, dimerization, or different types of ring degradations are elaborated here. In some cases, the observed microbial transformations mimic mammalian and/or plant metabolism. This review recognizes Norman Farnsworth, who through his fascination and hard work in pharmacognosy has fostered the excitement of discovery by numerous students and faculty far and beyond the halls of the University of Illinois at Chicago. It is with grateful thanks for these efforts that we dedicate this review to him.

Introduction

As integral constituents of the diet, flavonoids may exert a wide range of beneficial effects on human health, including protection against cardiovascular disease and certain forms of cancer.[1] Recent studies have shown diverse physiological and pharmacological activities of these natural compounds such as estrogenic,[2] antilipoperoxidant,[3] antitumor,[1] antiplatelet,[4] antiviral,[5] antifungal,[6] antibacterial,[7] antihemolytic,[8] anti-ischemic,[9] antiallergic, and anti-inflammatory.[10−12] These natural products also inhibit the activities of an array of enzymes including lipooxygenase, cyclooxygenase,[13,14] monooxygenase,[15] xanthine oxidase,[16] mitochondrial ATPase, succinoxidase and NADH-oxidase,[17,18] phospholipase A_2,[10] EGF receptor kinase, protein kinase C, MAP kinase,[18,19] topoisomerase II and cGMP, cAMP phosphodiesterase,[20] aldose reductase,[21] reverse transcriptase, and DNA polymerase.[22] Flavonoids likely produce such biological effects through their free radical-scavenging antioxidative activities[16,23] and metal ion-chelating abilities.[24] Some flavonoids are more potent than ascorbic acid and tocopherols in scavenging reactive oxygen species.

The basic flavonoid structure contains the flavan nucleus, which consists of 15 carbon atoms derived from a $C_6-C_3-C_6$ skeleton (**1**)[25] (Figure 1). Different classes of flavonoids are distinguished by additional oxygen-heterocyclic rings, by positional differences of the B-ring, and by hydroxyl, methyl, isoprenoid, and methoxy groups distributed in different patterns about the rings. Skeletons of some common flavonoids such as the chalcone (**2**), flavone (**3**), flavonol (**4**), flavanone (**5**), catechin (**6**), isoflavone (**7**), and isoflavanone (**8**) types are shown in Figure 1. Thousands of flavonoids have been reported in nature. Generally, flavonoids are present as glycosides in vacuoles of flowers, leaves, seeds, stems, or roots. Flavonoid aglycones, especially simple and polymethylated flavonoids, occur as farinose exudates or wax on the leaves, barks, and buds of species of *Primula*,[26] *Aesculus*,[27] *Pityrogramma*, *Cheilaanthes*, and *Notholaena*,[28] or as crystals in the cells of cacti.[29]

In mammals, flavonoids such as quercetin,[30] naringin,[31] (−)-epigallocatechin gallate,[32] (+)-catechin (**6**),[33] genistein,[34] and daidzein[35] are observed commonly in urine or in bile as glucuronide or sulfate conjugates or methyl ethers. (+)-Catechin is converted in the liver to 3′-*O*-methyl-(+)-catechin glucuronide, a major metabolite in both the bile and the urine of the rat.[36] 3′-*O*-Methyl-(+)-catechin is also found as a major (+)-catechin metabolite in human urine.[37] (+)-Catechin was degraded by ring fission to hydroxyphenyl-γ-valerolactone in the rat[38] and in man.[39] Moreover, several flavonoids were substrates for rat liver microsomal cytochrome P450.[40]

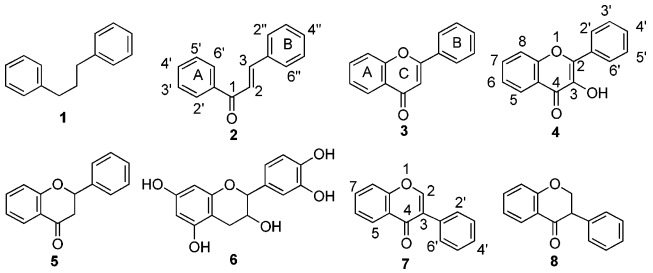

Figure 1. Skeletons, numbering, and lettering of chalcones, flavonoids, and isoflavonoids.

Microbial and Enzymatic Transformations of Flavonoids

Considerable time has lapsed since the literature of microbial and enzymatic transformations of flavonoids was summarized and reviewed.[41,42] Reactions of interest reviewed included a variety of oxidations, reductions, conjugations, ring cleavage, and deglycosylations. Recent work covered in this review has been driven by several concepts. These have included the use of biocatalysts to convert abundant prototype flavonoids to rarer products; the use of microorganisms as models for mammalian, plant, and soil metabolic transformations of flavonoids;[43−45] and their use as probes of the various mechanisms involved in enzymatic hydroxylations, methylations, and carbon−carbon bond formations. Exciting use of metabolic engineering of plant biosynthetic enzymes into microorganisms extends the field of biotransformations into new realms of possibility. The focus of this review is on microbial and enzymatic transformations of flavonoids. Occasional mention is given of comparable biotransformations observed in microorganisms and in mammals, plants, or plant cell cultures. This review is divided into sections on biotransformations of chalcones, isoflavones, catechins, and flavones.

Chalcones. The possible synthesis of $C_6-C_3-C_6$ chalcone precursors was investigated by using recombinant benzaldehyde

[⊥] Dedicated to Dr. Norman R. Farnsworth of the University of Illinois at Chicago for his pioneering work on bioactive natural products.

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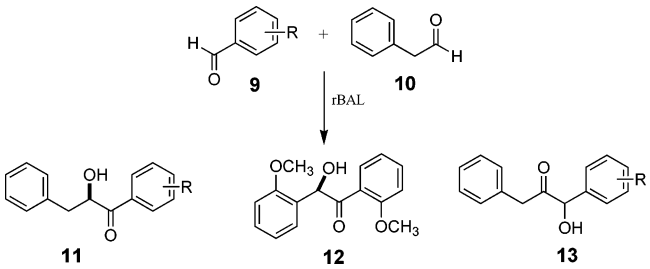

Figure 2. BAL-catalyzed phenylacetaldehyde and benzaldehyde condensation (R = 2-MeO; 2,3-DiMeO; 2,4-DiMeO; 2,5-DiMeO; 3,5-DiMeO).

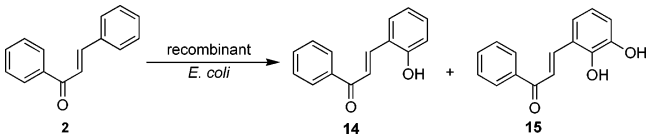

Figure 3. Chalcone (**2**) and hydroxylated metabolites by recombinant *E. coli* expressing biphenhyldioxygenase and dihydrodiol dehydrogenase.

lyase (BAL, EC 4.1.2.38).[46] BAL, a thiamine pyrophosphate (TPP)-dependent enzyme from *Pseudomonas fluorescens* Biovar I, catalyzes cleavage of the alpha-ketol carbon−carbon bond of benzoin to form two benzaldehydes.[46] BAL also catalyzes the reverse acyloin condensation of certain benzaldehydes, resulting in the synthesis of [*R*]-benzoins.[47] Recombinant BAL was used to catalyze mixed acyloin condensations of a series of methoxybenzaldehydes (**9**) and phenylacetaldehyde (**10**) to enantiomerically pure 2-hydroxy-1,3-diphenylpropan-1-ones (**11**), *o*-anisoins (**12**), and 1-hydroxy-1,3-diphenylpropan-2-ones (**13**) (Figure 2).[48] *R* absolute configurations of chiral centers were established by CD spectroscopy. [*R*]-Hydroxydihydrochalcones and 1-hydroxy-1,3-diphenylpropan-2-ones are valuable synthons for chemoenzymatic syntheses of flavonoids. The relaxed specificity of BAL enabled the synthesis of a variety of acyloin products. In general, trimethoxybenzaldehydes were poor substrates, while dimethoxy benzaldehydes and especially those substituted in the *meta* position gave fewer products in better yields.

Few microbial transformation studies have been reported directly on chalcones. The unsubstituted chalcone **2** was converted to 2″-hydroxychalcone (**14**) and 2″,3″-dihydroxychalcone (**15**) (Figure 3) in 25% and 59% yields, respectively, by *Escherichia coli* carrying modified biphenyl dioxygenase [*bphA1(2072)A2A3A4*] and dihydrodiol dehydrogenase genes (*bphB*).[49]

Hydroxylated chalcone metabolites also have been reported when flavanone undergoes ring opening. Udupa et al.[50] obtained 2′-hydroxy- and 2′,4″-dihydroxychalcones and dihydrochalcones when (±)-flavanone (**5**) was transformed by *Gibberella fujikuroi*. In this early work, flavanone carbonyls were stereospecifically reduced to alcohols by this fungus. Similarly, Abul-Hajj et al. also reported 2′-hydroxydihydrochalcone, 2′,4″-dihydroxydihydrochalcone, 2′,3″,4″-trihydroxydihydrochalcone, and 2′,5′-dihydroxydihydrochalcone as metabolites of (±)-flavanone by several microorganisms.[51]

A series of microorganisms were screened for their abilities to mimic plant metabolism in cyclizing chalcones to flavonoids. *Aspergillus alliaceus* UI 315 cyclized 2′-hydroxy-2″,3″-dimethoxychalcone (**16**) to three flavanones, **17**, **18**, and **19**, and to 3″-*O*-demethylated (**20**) and 3″-*O*-demethylated-3′-hydroxylated (**21**) chalcones (Figure 4).[52] Patterns of metabolites obtained from **16** with *A. alliaceus* cultures were dramatically altered when cytochrome P450 (CYP450) inhibitors SKF525A, metyrapone, and phenylthiocarbamide were included in culture media. These inhibitors revealed that as many as three different CYP450 enzyme systems were involved in chalcone biotransformations. One enzyme catalyzed chalcone cyclization, forming **17**, **18** and **19**; a second CYP450 was responsible for catalyzing 3″-*O*-dealkylation of **16** and **17** to chalcone **20** and flavanone **18**, respectively, while a third CYP450 catalyzed 3′-chalcone hydroxylation of **20** to **21**.

Flavanones obtained from *A. alliaceus* reactions were racemic, whereas similar products formed in plants are not.[52] In plants, chalcone isomerase catalyzes stereospecific chalcone cyclization to flavanones by an ionic 1,4-Michael addition with the α,β-unsaturated carbonyl functionality. Sanchez and Rosazza speculated that the enzyme system of *A. alliaceus* catalyzed a nonstereospecific, radical-based, intramolecular cyclization of chalcones to flavanones. One earlier study on microbial transformations of xanthohumol (**22**) by *Pichia membranifaciens* gave flavanone (**23**) plus altered chalcones **24** and **25** in the only other example of a microbial chalcone cyclization (Figure 5).[53]

Aurones were obtained when differently substituted chalcones were biotransformed by *A. alliaceus*. 4′,2″,4″-Trihydroxychalcone (isoliquiritigenin, **26**) was hydroxylated at position-3′ to form butein (**27**), which was cyclized to the aurone product sulfuretin (**30**) (Figure 6) (unpublished results). Inhibition experiments showed that initial C-3′ hydroxylation of **26** to **27** was catalyzed by a CYP450 enzyme system. Partially purified *A. alliaceus* polyphenol oxidase, a catechol oxidase, cyclized **27** to **30**. Thus, *A. alliaceus* uses a two-step process for aurone synthesis much like the plant biosynthetic pathway suggested by Nakayama et al.[54] In the first step, cytochrome P450 hydroxylates **26** at position 3′, giving **27**, while the ring-forming step that produces the aurone is catalyzed by a catechol oxidase, likely via an *o*-quinone intermediate (**28**).

Metabolic engineering in a common yeast, of components of the phenylpropanoid pathway, was used to prepare naringenin (**33b**), a central biosynthetic precursor of many flavonoids.[55] Genes for phenylalanine ammonia lyase (PAL) from *Rhodosporidium toruloides*, 4-coumarate:coenzyme A (CoA) ligase (4CL) from *Arabidopsis thaliana*, and chalcone synthase (CHS) from *Hypericum androsaemum* were introduced into two *Saccharomyces cerevisiae*

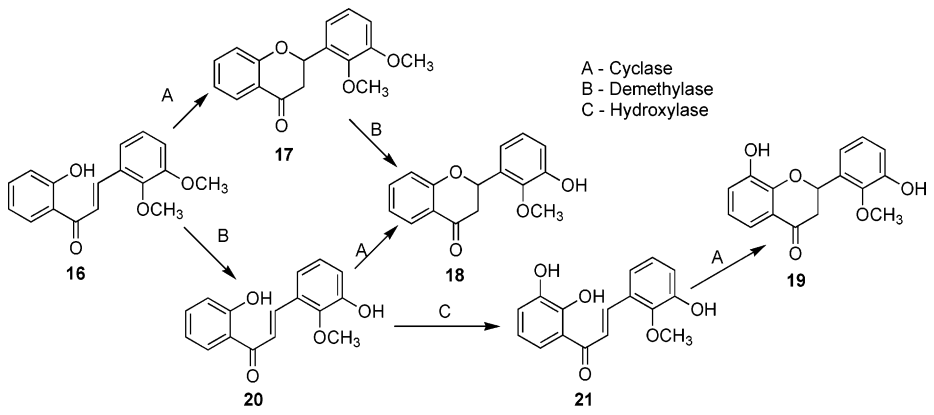

Figure 4. Biocatalytic pathway for the conversion of **16** to flavanones and altered chalcones by *A. alliaceus* UI 315.

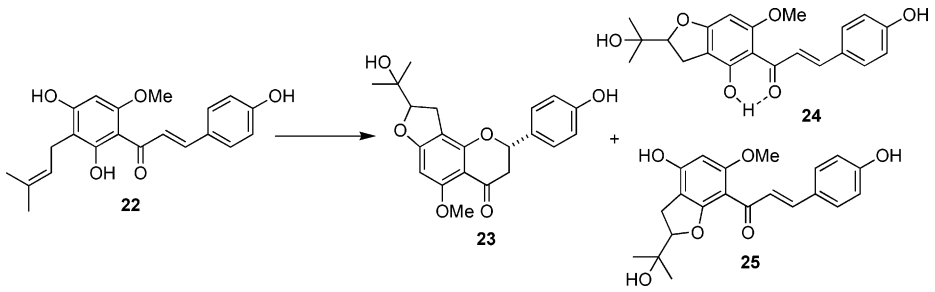

Figure 5. Structure of **22** and its metabolites formed by *Pichia membranifaciens*.

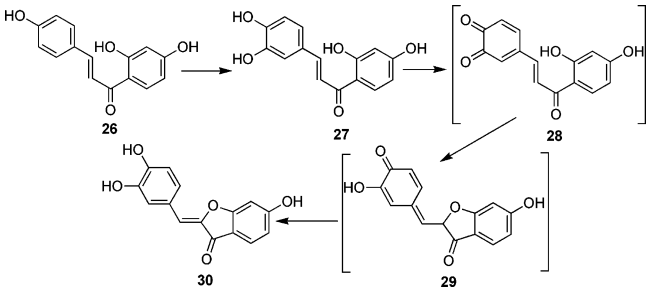

Figure 6. Proposed pathway for the biotransformation of isoliquiritigenin (**26**) to butein (**27**) and sulfuretin (**30**) by *A. alliaceus*.

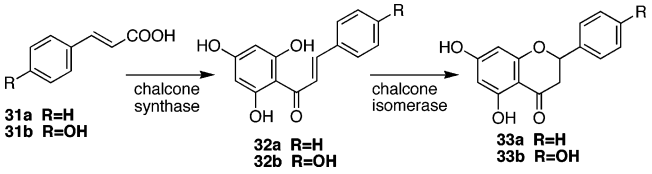

Figure 7. Flavonoid biosynthesis in recombinant *S. cereviseae* expressing phenyl-propanoid pathway enzymes.

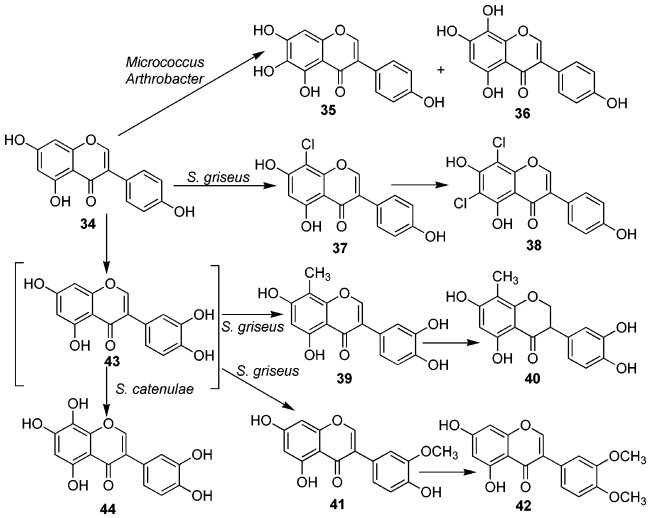

Figure 8. Biotransformations of genistein (**34**) by *S. griseus*, *S. catenulae*, and *Micrococcus* sp. and *Arthrobacter* sp. isolated from tempe.

strains. Besides its PAL activity, the recombinant PAL enzyme showed tyrosine ammonia lyase activity, which enabled the biosynthesis of naringenin (**33b**) without introducing cinnamate 4-hydroxylase (C4H). 4CL catalyzed the conversion of both *trans*-cinnamic acid (**31a)** and *p*-coumaric acid (**31b**) to their corresponding CoA products, which were further converted to pinocembrin chalcone (**32a**) and naringenin chalcone (**32b**) by CHS. These chalcones were cyclized by chalcone isomerase (CI) to pinocembrin (**33a**) and naringenin, respectively (Figure 7). The yeast AH22 strain coexpressing PAL, 4CL, and CHS produced approximately 7 mg L^{-1} of naringenin and 0.8 mg L^{-1} of pinocembrin. Additional dihydrochalcones were identified as minor byproducts in the recombinant biosynthetic system.

Isoflavones. Isoflavonoids are among the largest groups of flavonoids commonly found in members of the Leguminosae plant family.[42] Genistein and daidzein are the most abundant aglycones found in soybeans,[56] where they occur as their 7-(6-*O*-acetyl)-glucosides known as genistin and daidzin, respectively. The abundance of compounds such as genistein and daidzein render them ideal as starting materials from which rarer isoflavones might be prepared by microbial transformation.

Micrococcus (I) or *Arthrobacter* species isolated from "tempe", a traditional Indonesian soybean-derived food, hydroxylated genistein (**34**) at the 6- or 8-position to form 5,6,7,4′-tetrahydroxyisoflavone (**35**) and 5,7,8,4′-tetrahydroxyisoflavone (**36**) (Figure 8). Both strains also transformed biochanin A, the 4′-*O*-methyl equivalent of genistein, to 4′-methoxy-5,7,8-trihydroxyisoflavone, whereas only strain I converted biochanin A to 4′-methoxy-5,6,7-trihydroxyisoflavone.[57] This study was important in view of the pronounced biological activity and possible nutritional value of these isoflavones in tempe as a food product.

Genistein halogenations were observed when *Streptomyces griseus* ATCC 13273 was cultivated in a soybean-meal-containing medium.[58] Along with genistein, 8-chlorogenistein (**37**) and 6,8-dichlorogenistein (**38**) were isolated and characterized (Figure 8). The presence of **34** in soybean meal used in culture media was confirmed by extraction, isolation, and spectroscopic identification. *S. griseus* grown in medium without soybean meal produced no chlorinated isoflavonoids. *S. griseus* gave both **37** and **38** when incubated with genistein, showing that these isoflavonoids are products of microbial halogenation.

S. griseus grown with ^{14}C-acetate and ^{14}C-phenylalanine, biosynthetic precursors for flavonoids in plants,[46,59] produced no radiolabeled **34**. This work demonstrated that isoflavonoids were not biosynthesized from expected biosynthetic precursors by *S. griseus* and that the reported isolation of such compounds from other Streptomycetes was largely fermentation medium artifacts.

S. griseus and *S. catenulae* strains transformed genistein (**34)** into a variety of hydroxylated and methylated metabolites including new isoflavones **39**, **40**, **41**, and **44** and the known 5,7-dihydroxy-3′,4′-dimethoxyisoflavone (**42**).[60] All biotransformation products contained an *ortho*-dihydroxy-substituted ring B. Metabolite structures suggested that the pathways for genistein biotransformations by *S. catenulae* and *S. griseus* required initial 3′-hydroxylation to form the unisolated catechol **43**. Carbon methylation of **43** at C-8 gave **39**, while reduction of the C_2-C_3 double bond gave **40**. In *S. griseus*, 3′- and 4′-*O*-methylation of putative intermediate **43** gave **41** and **42**. *S. catenulae* gave the very unusual bis-catechol **44** by further hydroxylation of **43** (Figure 8).

Genistein, a substrate for *S. griseus*, induces soluble $CYP450_{SOY}$ in the same organism.[61] By hydroxylation, presumably by CYP450, *S. griseus* produced a catechol that appeared to be a requisite for methylation either at carbon or at phenolic oxygens. Methylations observed with genestein biotransformation resemble the equivalent mammalian, phase II conjugation metabolic reaction.[62,63] Because the methylation reactions with genistein were relatively unusual,

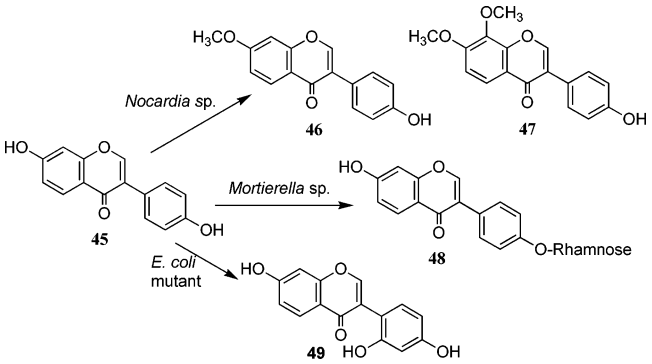

Figure 9. Metabolism of daidzein (**45**) by *Nocardia* sp., *Mortierella isabellina*, and recombinant *E. coli* containing biphenyl dioxygenase.

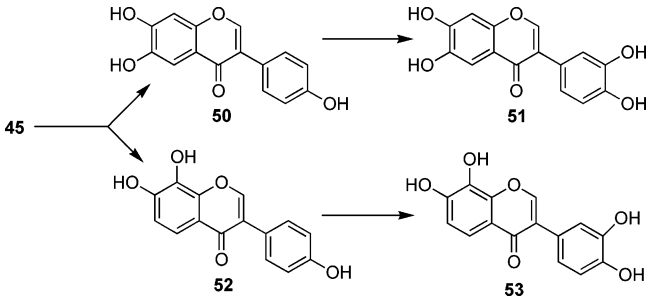

Figure 10. Hydroxylations of daidzein (**45**) by organisms isolated from tempe.

efforts were made to determine whether *S. griseus* contained a methyltransferase similar to that known in mammals.

A soluble (100000*g* supernatant) methyltransferase catalyzing the transfer of the methyl group of *S*-adenosyl-L-methionine to catechols was present in *S. griseus* cell extracts.[64] The enzyme was purified 141-fold by precipitation with ammonium sulfate and ion exchange chromatography, showing an apparent molecular mass of 36 kDa for both the native and denatured protein. Catechol, caffeic acid, and 4-nitrocatechol were methyltransferase substrates. As with mammalian counterparts, homocysteine was a competitive inhibitor of *S*-adenosyl-L-methionine, and sinefungin and *S*-adenosylhomocysteine inhibited methylation. This was the first report of a bacterial COMT enzyme system.

Daidzein (**45**), the second most abundant soybean isoflavone aglycone, was both hydroxylated and methylated by *Nocardia* species NRRL 5646 to 7-methoxy-4′-hydroxyisoflavone (isoformononetin) (**46**) and 7,8-dimethoxy-4′-hydroxyisoflavone (**47**) (Figure 9) (unpublished results). The fungus *Mortierella isabellina* ATCC 38063 converted **45** to daidzein-4′-rhamnopyranoside (**48**), a new natural product (Figure 9) (unpublished results). Daidzein was converted to 7,2′,4′-trihydroxyflavone (**49**) by a recombinant *E. coli* strain containing genes for biphenyl-2,3-dioxygenase (BphA) and biphenyl-2,3-dihydrodiol-2,3-dehydrogenase (BphB).[65] Dehydration of a presumed dihydrodiol intermediate (not shown) apparently favors removal of the introduced 3′-hydroxyl group in producing **49**. In a related study, 7-hydroxyisoflavone was converted to 7,1′,2′-trihydroxyisoflavone and 2,3-dihydro-7,1′,2′-trihydroxyisoflavone in 47% and 56% yields, respectively, by *E. coli* cells expressing the BphA and BphB.[49] Miyazawa et al. used *A. niger* to convert 7,4′-dimethoxyisoflavone to 6-hydroxy-7,4′-dimethoxyisoflavone by 6-hydroxylation and daidzein (**45**) by 7,4′-demethylation (not shown).[66] Diacetoxydaidzein was hydrolyzed to afford daidzein by the same microorganism.

Five tempe-derived bacterial strains transformed daidzein (**45**) to 6,7,4′-trihydroxyisoflavone (**50**) and further to 6,7,3′,4′-tetrahydroxyisoflavone (**51**) (Figure 10).[67] Three strains oxidized **45** to 7,8,4′-trihydroxyisoflavone (**52**) and further to 7,8,3′,4′-tetrahydroxyisoflavone (**53**). In addition, two isolates also transformed daidzein to the unusual trihydroxy-ring-A derivative, 6,7,8,4′-tetrahydroxyisoflavone.

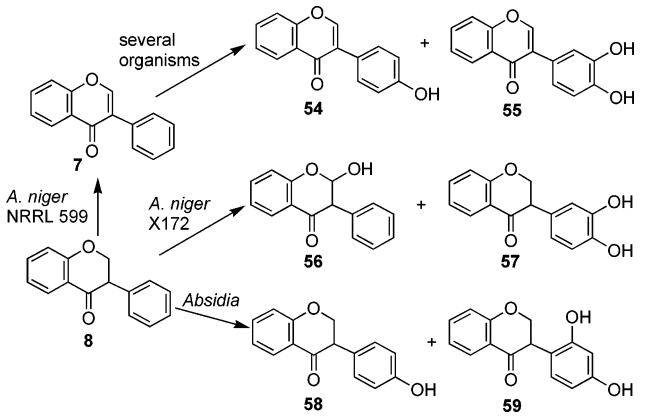

Figure 11. Structures of metabolites obtained from unsubstituted isoflavone (**7**) and isoflavanone (**8**).

The Gram-positive, human fecal anaerobe, *Eubacterium ramulus*, cleaved the C-rings of daidzein and genistein to enantiomerically pure 1-(2,4-dihydroxyphenyl)-2-(4-hydroxyphenyl)-1-propanone (ee 90%) and 2-(4-hydroxyphenyl) propionic acid (ee 98%), respectively (not shown).[68] In this C-ring cleavage, daidzein was not further degraded to the propionic acid derivative. Biotransformation kinetics indicated that the genistein C-ring was more susceptible to bacterial degradation than that of daidzein.

Biotransformations of unsubstituted isoflavone (**7**)[69] and isoflavanone (**8**)[51] were studied to elaborate isoflavonoid metabolic pathways possibly relating to their biological activities and potential toxicities.[70] With **7**, *Aspergillus niger* X172, *Cunninghamella blakesleeana* ATCC 8688A, *Helicostylum piriformi* QM 6945, and *Penicillium purpurogenum* U-193 gave 4′-hydroxyisoflavone (**54**) and 3′,4′-dihydroxyisoflavone (**55**) as the major products (Figure 11), mimicking similar hydroxylations in mammals.[43,44] Isoflavanone (**8**) was converted to 2-hydroxyisoflavanone (**56**) and 3′,4′-dihydroxyisoflavanone (**57**) by *A. niger* X172, whereas *Absidia blakesleeana* NRRL 1306 gave 4′-hydroxyisoflavanone (**58**) and 2′,4′-dihydroxyisoflavanone (**59**). *A. niger* NRRL 599 converted **8** to **7** by dehydrogenation.

Ibrahim and Abul-Hajj also studied biotransformations of flavone and flavanone, which are described later in this review. A model postulated for the interaction of flavonoids at a hydroxylase active site suggested that the ether and carbonyl oxygen atoms of the chromone ring were bound to hydrophilic sites on an enzyme surface, orienting ring B to the hydroxylating site of the enzyme.[51]

E. coli expressing biphenyl-2,3-dioxygenase (BphA) and biphenyl-2,3-dihydrodiol 2,3-dehydrogenase (BphB) converted 7-hydroxyisoflavone (**60**) to 7,3′,4′-trihydroxyisoflavone (**61**), and 7-hydroxy-8-methylisoflavone (**62**) through **63** to 7,2′,3′-trihydroxy-8-methylisoflavone (**64**), respectively (Figure 12).[65] In a very unusual microbial defluorination reaction, 2′-fluoro-7-hydroxy-8-methylisoflavone (**65**) gave **64** via presumed hydrodiol intermediate **66**, which was subsequently aromatized by loss of HF.

Eubacterium limosum (ATCC 8486), a strict anaerobe from the human intestinal tract, *O*-demethylated formononetin (**67a**), biochanin A (**67b**), and glycitein (**67c**) to products **68a**, **68b**, and **68c**, respectively (Figure 13).[71] *O*-Demethylase activity was cell-associated and was not detected in the extracellular fraction of bacterial cultures. *O*-Demethylated products formed by this intestinal microorganism were all more potent phytoestrogens than the starting materials using estrogen-receptor-based assays.[72]

Mackenbrock et al. studied biotransformations of isoflavones with special interest on hydroxylation adjacent to an existing methoxy group.[73] Formononetin (**67a**) and biochanin A (**67b**) were converted into pratensein (**69a**) and calycosin (**69b**), respectively, by 3′-

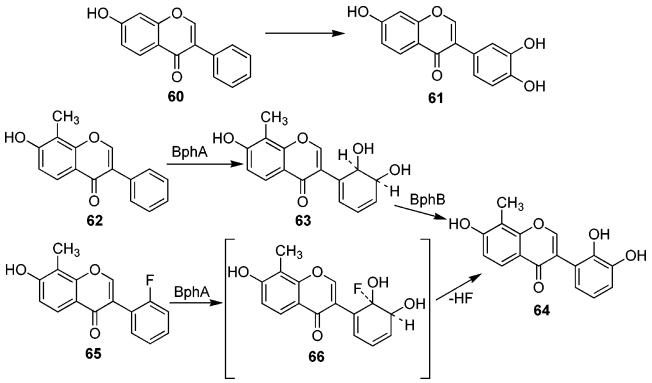

Figure 12. Biotransformations of selected isoflavones by recombinant *E. coli* containing BphA and BphB.

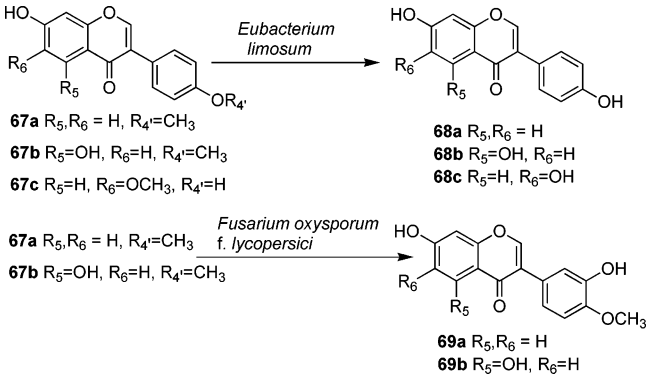

Figure 13. *O*-Dealkylation and 3′-hydroxylations of isoflavones.

hydroxylation by *Fusarium oxysporum* f. *lycopersici*. Genistein, daidzein, 5,7,4′-trimethoxyisoflavone, and 6,7-dihydroxy-4′-methoxyisoflavone (texasin) were also used as substrates. Of these, only genistein was converted to the 3′-hydroxylated product, orobol, in 10% yield. It was concluded that 3′-hydroxylation of isoflavones by *F. oxysporum* f. *lycopersici* mainly proceeds with 4′-methoxy-7-hydroxy derivatives, and the order of the rate of this reaction is biochanin A ≫ formononetin ≫ genistein. This reaction was used for quantitative conversion of ^{14}C-labeled isoflavones to their hydroxylated products.

Catechins. Catechins are the basic structural units of condensed tannins. They belong to the class of flavan-3-ols and are found in a wide variety of vegetables, herbs, and teas. Catechins are protective against cancer and inflammatory and cardiovascular diseases,[10−12] presumably because of their antioxidant activities and ability to scavenge free radicals.[16,23] As polyphenols, catechins are susceptible to enzymatic and nonenzymatic oxidations, giving rise to a variety of dimeric, oligomeric, and polymeric products.[74,75] Oxidations of phenols by grape polyphenol oxidase (PPO),[76] hydrogen peroxide (H_2O_2)-dependent peroxidases,[77,78] and copper oxidases[77,78] have been described in the literature.

In studies on the bacterial degradation of catechins, a *Pseudomonas* sp. was isolated from rat feces and grown on (+)-catechin.[79,80] Cell-free extracts prepared from this organism catalyzed initial 8-hydroxylation of taxifolin (**70**) to the ring-A, 7,8-catechol, dihydrogossypetin (**71**) (Figure 14). Subsequent *ortho*-dioxygenase A-ring cleavage through putative intermediate **72** gave oxaloacetic acid (**73**) and 5-(3,4-dihydroxyphenyl)-4-hydroxy-3-oxovalero-δ-lactone (**74**). Synthesis of related phenylvalerolactones confirmed the structure of this lactone.

Considerable literature describes the formation of polymeric products when (+)-catechin (**6**) is subjected to chemical oxidation, autoxidation, or enzymatic oxidation. Oxidation product structure determinations are challenging because of difficulties in metabolite purification and in spectroscopic correlations by NMR spectroscopy.

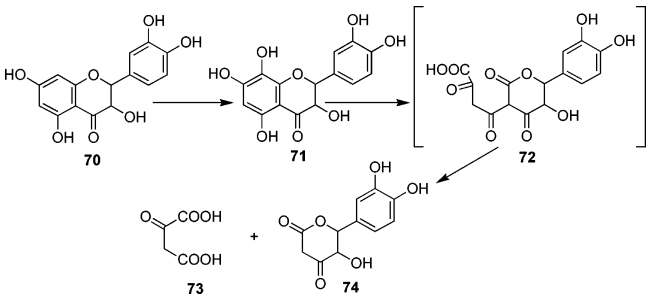

Figure 14. A-ring degradation of taxifolin (**70**).

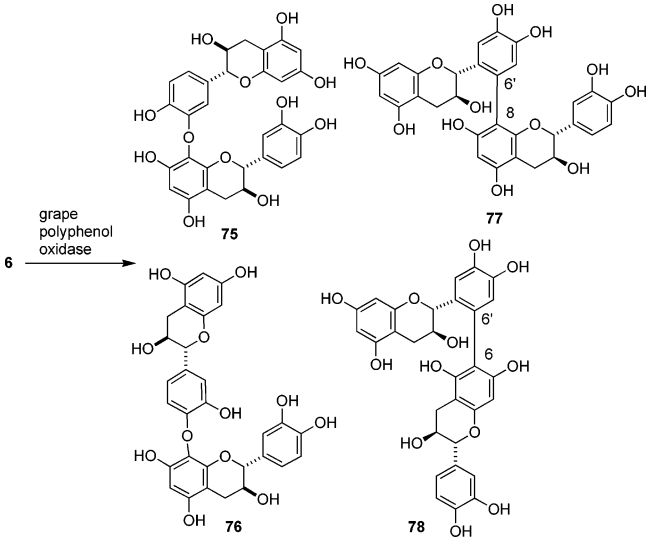

Figure 15. Dimeric metabolites obtained by grape polyphenol oxidase oxidation of (+)-catechin (**6**).

When exposed to grape polyphenol oxidase at pH 3 and pH 6, (+)-catechin (**6**) gave complex mixtures of dimeric products that were isolated and characterized by mass spectrometry and by NMR spectroscopic analysis (Figure 15).[76] Structures were suggested as biphenyl-ether dimers (either **75** or **76**), carbon−carbon dimers (either **77** or **78**), and two yellow dimeric pigments, one of which was identified spectroscopically as dehydrodicatechin (not shown). Dimeric structures such as these posed difficulties in assigning specific sites of dimeric linkages, thus forcing the conclusion that either **75** or **76**, or **77** or **78**, was the isolated product. Products similar to these obtained by $K_3[Fe(CN)_6]$[81] or enzymatically with hydrogen peroxide-dependent peroxidase[82] also have been characterized.

Dimers formed by H_2O_2-dependent, horseradish peroxidase (HRP)-catalyzed oxidative coupling of (+)-catechin (**6**) gave three novel dimeric products, **79**−**81** (Figure 16).[83] All three dimers were purified and characterized unambiguously by MS and NMR spectroscopic analyses. Spectroscopic evidence showed that HRP−H_2O_2 dimers were linked through position 8″ of the A-ring of one catechin moiety to C-5′ of ring B in **79** and **80** and to C-2 of ring C in **81**. The unusual catechin dicarboxylic acid dimer **80** was obtained by *ortho*-cleavage of the E-ring of **79**.

When treated with mushroom tyrosinase in the presence of excess phloroglucinol, (+) catechin (**6**) was converted to the adduct (2*R*,3*S*)-2,3-*trans*-6′-(2″,4″,6″-trihydroxyphenyl)-3′,4′,5,7-tetrahydroxyflavan-3-ol (**82**) as the only product in 46% yield (Figure 17).[84] Coupling of phloroglucinol with **6** was presumed to occur via a reactive 3′,4′-*o*-quinone intermediate formed by catechin B-ring oxidation. Other A-ring monophenolic flavan-3-ols including fisetinidol and mesquitol also gave tyrosinase-mediated, phloroglucinol-coupled products. With the copper oxidase *Rhus vernicifera* laccase, (+)-catechin was converted into two new hydroquinone adducts, **83** and **84** (Figure 17).[83] Hydroquinone served as both a

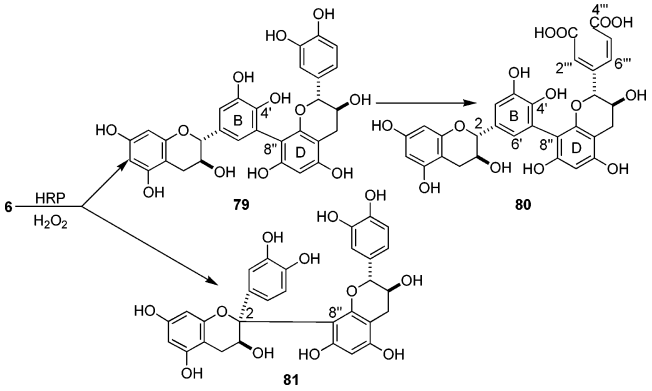

Figure 16. Oxidative dimerization of (+)-catechin (**6**) by horseradish peroxidase-H_2O_2.

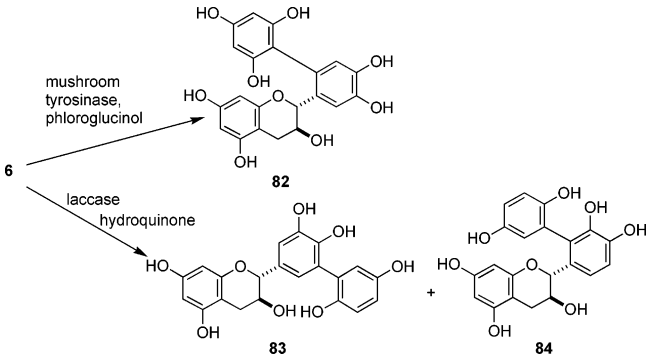

Figure 17. Mushroom tyrosinase (phloroglucinol) and laccase (hydroquinone) oxidations of (+)-catechin (**6**).

shuttle oxidant and a reactant by coupling at C-2′ and C-5′ of the catechin B-ring during laccase oxidations.

HRP and laccase oxidation products were compared to D,L-α-tocopherol and (+)-catechin (**6**) for their abilities to inhibit iron-induced lipid peroxidation in rat brain homogenates and Fe^{3+}-ADP/NADPH in rat liver microsomes, as measured by the intensity of thiobarbituric acid reactive substance.[83] All metabolites exhibited antilipid peroxidation with IC_{50} values ~2−8 times higher than those of standard compounds. These results show that these metabolites have antioxidant properties slightly better than D,L-α-tocopherol and catechin themselves. This is interesting in that although the products obtained by peroxidase and laccase enzyme oxidations were formed by radical processes, they retain slightly enhanced antioxidant activities. Characteristic reaction products may prove to be novel markers for (+)-catechin antioxidant reactions in living systems.

Oxidations of catechin (**6**) in the presence of glutathione by tyrosinase, horseradish peroxidase, and hydrogen peroxide and rat liver microsomes gave mixtures of mono-, di-, and triglutathione conjugates and mono- and diglutathione conjugates of a catechin dimer.[85] A hydroxylated catechin glutathione conjugate was also detected. None of the metabolites were isolated, and structures were deduced by analysis of gas chromatography−mass spectrometry. Rat liver microsomes and NADPH or cumene hydroperoxide also catalyzed catechin−glutathione conjugate formation, a reaction that was prevented by benzylimidazole, a cytochrome P450 2E1 inhibitor. Catechin cytotoxicity toward isolated hepatocytes was also enhanced markedly by hydrogen peroxide or cumene hydroperoxide and was prevented by benzylimidazole, suggesting that catechin can be metabolically activated by P450 peroxidase activity to form cytotoxic quinoid species. The catechol B-ring of catechin was oxidized by tyrosinase to an *o*-quinone, which could be reduced back to catechin with potassium borohydride or reacted with GSH to form glutathione conjugates.

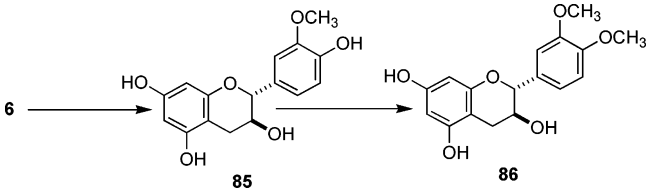

Figure 18. Methylations of (+)-catechin (**6**) by *S. griseus*.

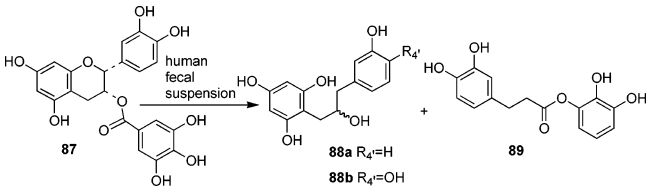

Figure 19. Metabolites of (−)-epigallocatechin-3-*O*-gallate (**87**) by human fecal suspensions.

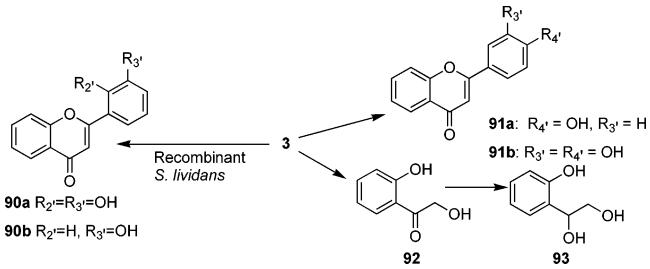

Figure 20. Structures of flavone (**3**) microbial transformation metabolites.

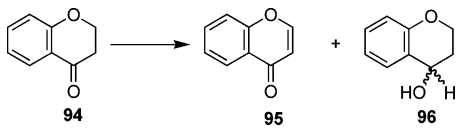

Figure 21. *Aspergillus niger* NRRL 599 biotransformations of chromanone (**94**).

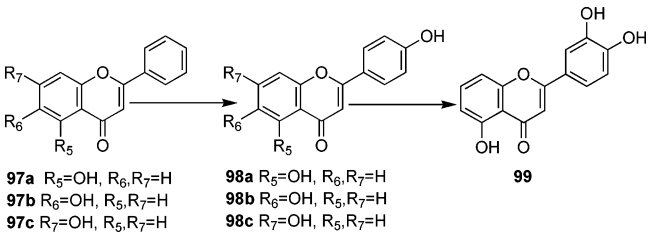

Figure 22. *S. fulvissimus* B-ring hydroxylations of flavones **97a**−**c**.

S. griseus transformed (+)-catechin (**6**) in stepwise fashion to 3′-*O*-methyl-(+)-catechin (**85**) and 3′,4′-*O*-dimethyl-(+)-catechin (**86**), both examples of phase II type metabolic transformation reactions (Figure 18).[86] *S. griseus* mimics mammalian catechin metabolism since **85** is a known mammalian metabolite. It is likely that *S. griseus* catechol-*O*-methyltransferase was responsible for methylation of catechin.[64]

A *Eubacterium* (*E.*) sp. isolated from human intestinal flora transformed various (3*R*)- and (3*S*)-flavan-3-ols to 1,3-diphenylpropan-2-ol derivatives by cleaving the C-ring of (3*R*)- and (3*S*)-flavan-3-ols.[87] This bacterium also dehydroxylated the B-ring 4′-positions of (3*R*)-flavan-3-ols, such as (−)-catechin, (−)-epicatechin, (−)-gallocatechin, and (−)-epigallocatechin, but not those of (3*S*)-flavan-3-ols.

Biotransformations of (−)-epicatechin-3-*O*-gallate (**87**) and related compounds by a human fecal suspension gave 15 metabolites, four of which had not been previously characterized.[88] These were identified as the two epimers of 1-(3′-hydroxyphenyl)-3-

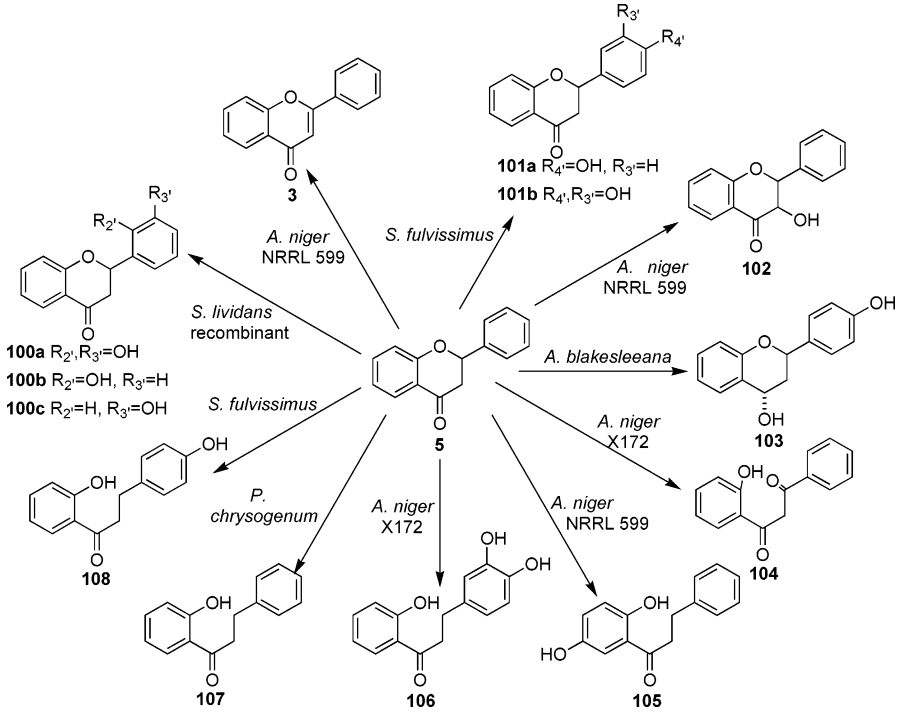

Figure 23. Microbial transformation products of unsubstituted flavanone (**5**).

(2″,4″,6″-trihydroxyphenyl)propan-2-ols (**88a**), 1-(3′,4′-dihydroxyphenyl)-3-(2″,4″,6″-trihydroxyphenyl)propan-2-ol (**88b**), and 2″,3″-dihydroxyphenoxyl-3-(3′,4′-dihydroxyphenyl)propionate (**89**) (Figure 19). Bacterial reaction sequences leading to these metabolites are obviously complex, involving C-ring fission, 5-dehydroxylation, and hydroxylation at position 8. (−)-Epicatechin, (−)-epigallocatechin, and their 3-*O*-gallates were extensively metabolized by human fecal suspensions, whereas the gallates resisted any degradation by rat fecal suspensions, even after prolonged incubation, showing differences in metabolic activity between intestinal bacterial mixtures from different species.

Flavones. Flavones, flavanols, and flavanones are among the largest groups of flavonoids. Chun et al.[89] constructed a recombinant *Streptomyces lividans* strain containing a *bphA1(2072)A2A3A4* gene cluster coding for a shuffled biphenyl dioxygenase holoenzyme with broad substrate specificity. The *bphA1(2072)* hybrid gene encoding the large subunit of an iron−sulfur protein was generated by DNA shuffling using bphA1 of *Pseudomonas pseudoalcaligenes* KF707 and bphA1 of *Burkholderia cepacia* LB400. The bphA2, bphA3, and bphA4 genes from *P. pseudoalcaligenes* KF707 encoded the small subunit of an iron−sulfur protein, ferredoxin, and a ferredoxin reductase, respectively. Biotransformation of 1 mM flavone (**3**) by this recombinant *S. lividans* strain gave 2′,3′-dihydroxyflavone (**90a**) and 3′-hydroxyflavone (**90b**) in 13% and 2.4% yields, respectively, within 24 h (Figure 20). The 6-hydroxyflavone analogue was efficiently converted into 6,2′-dihydroxyflavone.

Biotransformations of unsubstituted flavone (**3**)[69] by many fungi including species of *Aspergillus*, *Cunninghamella*, *Helicostylum*, *Penicillium*, and *Linderina* and a Streptomycete gave 4′-hydroxyflavone (**91a**) and 3′,4′-dihydroxyflavone (**91b**) as the major products. C-ring cleavage of **3** was a pathway among fungal species of *Absidia*, *Gongronella*, *Rhizopus*, *Manascus*, and *Gymnascella*. Products identified were *o*-hydroxyphenylhydroxymethyl ketone (**92**), while *Rhizopus nigricans* gave *o*-hydroxyphenylethanediol (**93**) by reduction of **92** (Figure 20). No stereochemistry was indicated in the ketone reduction reaction.

Ibrahim and Abul-Hajj examined 32 microorganisms for their abilities to catalyze biotransformations of chromone, chromanone, and several ring-A hydroxy-flavones.[90] Chromone itself, equivalent to the A- and C-rings of flavone substrates, was not transformed.

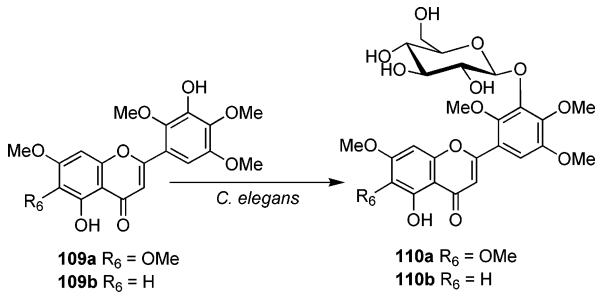

Figure 24. Glucosidation of psiadiarabin (**109a**) and 6-desmethoxypsiadiarabin (**109b**) by *C. elegans*.

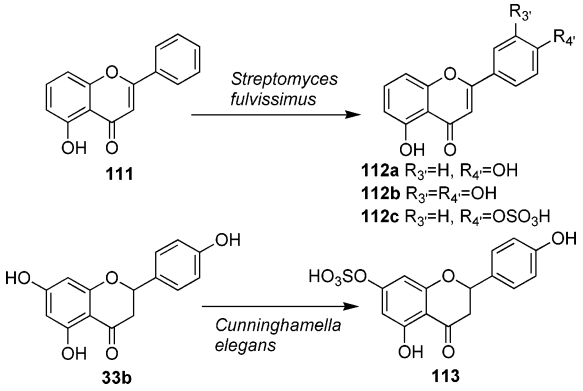

Figure 25. Microbial hydroxylations and sulfations of 5-hydroxyflavone (**111**) and sulfation of naringenin (**33b**).

On the other hand, *Aspergillus niger* NRRL 599 dehydrogenated chromanone (**94**) to chromone (**95**) and reduced the ketone of **94**, giving chroman-4-ol (**96**) of undefined stereochemistry (Figure 21).

In the same study, *Streptomyces fulvissimus* NRRL 1453B hydroxylated 5-hydroxy-(**97a**), 6-hydroxy-(**97b**), and 7-hydroxyflavone (**97c**) to their corresponding 4′-hydroxylated products (**98a**−**c**) (Figure 22). 5-Hydroxyflavone (**97a**) also gave the dihydroxylated 5,3′,4′-trihydroxyflavone (**99**). Yields and rates of 4′-hydroxylation were apparently dependent upon the distance between the C-4 carbonyl group and the hydroxyl group in ring A.

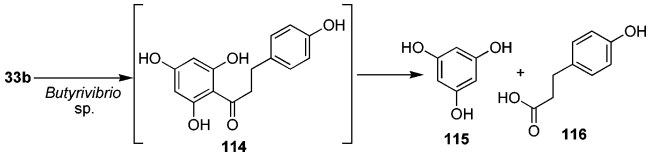

Figure 26. Rumen bacterial degradation of naringenin (**33b**).

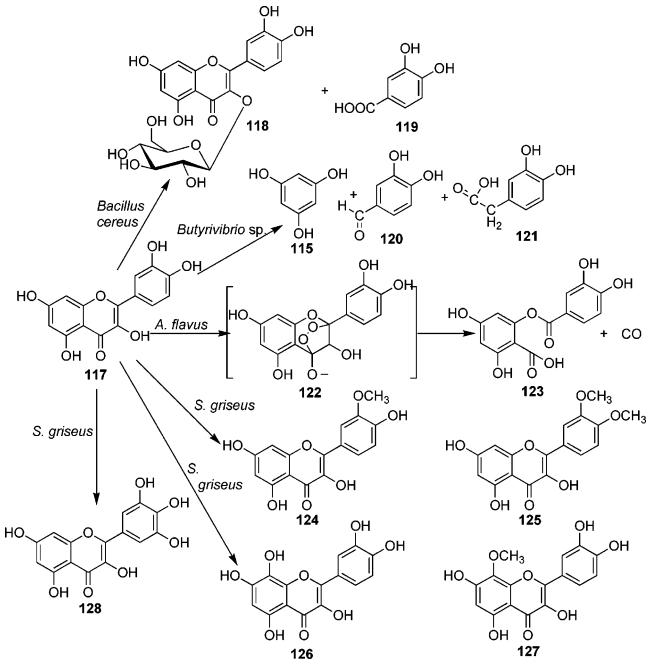

Figure 27. Microbial transformations of quercetin (**117**).

Unsubstituted flavanone (**5**) was transformed by a number of microorganisms, yielding a wide array of products (Figure 23). Recombinant *Streptomyces lividans* containing the biphenyl dioxygenase *bphA1(2072)A2A3A4* gene cluster transformed **5** to 2′,3′-dihydroxyflavanone (**100a**), 2′-hydroxyflavanone (**100b**), and 3′-hydroxyflavanone (**100c**) in 16%, 4.4%, and 4.6% yields, respectively.[89] 6-Hydroxyflavanone (not shown) was converted into 6,3′-dihydroxyflavanone.

Species of *Absidia*, *Aspergillus*, *Penicillium*, and *Streptomyces* transformed unsubstituted flavanone (**5**) into numerous metabolites obtained by hydroxylations at various positions, C-ring dehydrogenation, and ring-C cleavage (Figure 23).[51] These were characterized as flavone (**3**), 4′-hydroxyflavanone (**101a**), 3′,4′-dihydroxyflavanone (**101b**), 3-hydroxyflavanone (**102**), 4′-hydroxyflavan-4-α-ol (**103**), 2′-hydroxydibenzoylmethane (**104**), 2′,5′-dihydroxydihydrochalcone (**105**), 2′,3″,4″-trihydroxydihydrochalcone (**106**), 2′-hydroxydihydrochalcone (**107**), and 2′,4″-dihydroxydihydrochalcone (**108**). Results of this study clarified substrate specificities in microbial transformations of flavonoids, showing a preponderance of B-ring hydroxylations by microorganisms.

A number of studies afforded phase II flavonoid conjugates including phenolic glucosides and sulfates. Biotransformations of the highly oxygenated flavonoid psiadiarabin (**109a**) and its 6-desmethoxy analogue, 5,3′-dihydroxy-7,2′,4′,5′-tetramethoxyflavone (**109b**), for example by *Cunninghamella elegans* NRRL 1392 gave the corresponding 3′-*O*-α-D-glucosides (**110a** and **110b**) (Figure 24).[91] Glucosidation at the B-ring position 3′ is interesting because the phenolic moiety that undergoes conjugation is relatively hindered.

Sulfation reactions were also observed with bacterial and fungal transformations of flavones. Aromatic hydroxylation and sulfation of 5-hydroxyflavone (**111**) was observed with *Streptomyces fulvissimus*, leading to 5,4′-dihydroxyflavone (**112a**), 5,3′,4′-trihydroxyflavone (**112b**), and 5,4′-dihydroxyflavone-4′-sulfate (**112c**) (Figure 25).[92] Ibrahim reported the sulfation of naringenin (**33b**) to naringenin-7-sulfate (**113**) by *Cunninghamella elegans* NRRL 1392 in 23% yield.[93] Sulfation is a rare microbial transformation reaction, paralleling mammalian phase II metabolism.

Bovine rumen microorganisms rapidly degrade the flavonoid glycosides rutin, quercitrin, naringin, and hesperidin. A *Butyrivibrio* sp. C_3 isolated from the rumen hydrolyzed glycosidic bonds of the glycosides, liberating the aglycones. When naringenin was subjected to anaerobic growth in a rumen-fluid-containing medium and 0.5% naringin, the products naringenin (**33b**), phloroglucinol (**115**), and *p*-hydroxyphenylpropionic acid (**116**) were obtained (Figure 26).[94] The postulated sequence involves ring-C cleavage to **114** as the precursor of **115** and **116**.

Rao et al.[95] examined biotransformations of quercetin (**117**) by *Bacillus cereus*. This organism catalyzed 3-*O*-glucosylation, forming isoquercitrin (**118**) and protocatechuic acid (**119**), likely by initial C-ring cleavage (Figure 27).

Anaerobic degradation of the glycoside rutin by *Butyrivibrio* sp., the most active flavonoid-degrading organism isolated from bovine rumen, first gave quercetin (**117**), glucose, and rhamnose by sugar hydrolyses.[96] The aglycon **117** was further degraded to phloroglucinol (**115**), CO_2, 3,4-dihydroxybenzaldehyde (**120**), and 3,4-dihydroxyphenylacetic acid (**121**) (Figure 27). The products formed by *Butyrivibrio* sp. are similar to those found in the urine of rats fed quercetin.[97] Thus, the degradation mimics mammalian metabolism.

Aspergillus flavus transformed quercetin to carbon monoxide and a depside, 2-protocatechuoylphloroglucinolcarboxylic acid (**123**), through enzymatic oxygenation and cleavage of quercetin by quercetinase (Figure 27).[98] The mechanism of the reaction was studied by conducting reactions in an $^{18}O_2$ atmosphere and in $H_2{}^{18}O$. On the basis of mass spectrometric results, two oxygen atoms were incorporated into the depside from molecular oxygen and no oxygen was incorporated from water. One atom of $^{18}O_2$ was incorporated into the carboxyl group of **123** and the other into the ester carbonyl,

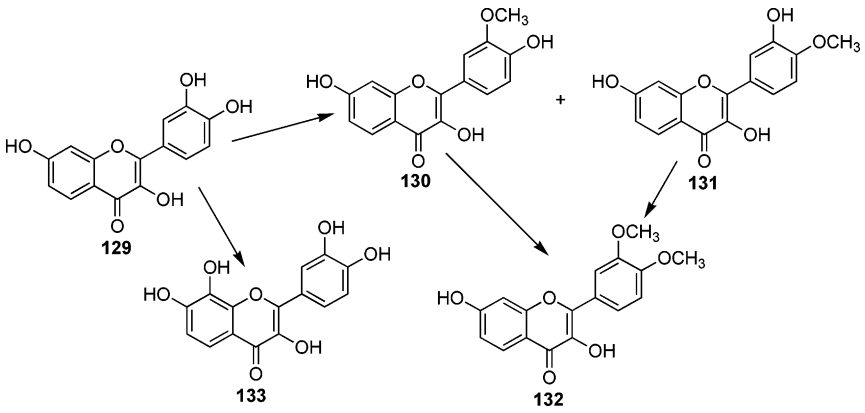

Figure 28. *Streptomyces griseus* metabolites of fisetin (**129**).

indicating that quercetinase activated and inserted both atoms of molecular oxygen as a dioxygenase via **122**.

With *Streptomyces griseus* (ATCC 13273), quercetin (**117**) afforded hydroxylated and/or methylated metabolites (**124**−**127**) (Figure 27).[86] The unusual triphenolic B-ring metabolite **128** was also obtained. Aromatic hydroxylation occurred at the A- and B-rings, and both mono- and dimethoxy metabolites were obtained.

In a similar fashion, *S. griseus* catalyzed A-ring hydroxylation of fisetin (**129**) to **133** and B-ring *O*-methylations giving mono- and dimethoxy products **130**−**132** (Figure 28).[86] With *S. griseus*, methylation was observed only in metabolites that contained catechol moieties. The *S*-adenosyl methionine-dependent, catechol-*O*-methyl transferase (COMT) from *S. griseus*[64] was the first COMT found in bacteria. Mammalian *O*-methyltransferases that are capable of methylating flavonoid catechols have been reported.[99] In mammals, *O*-methylation is a well-known detoxication pathway. *S. griseus* appears to mimic mammalian metabolism in producing monomethyl ethers from catechol substrates that are prone to oxidation and redox cycling. Further similarity to mammalian metabolism was seen in the *S. griseus* COMT-mediated methylation of quercetin and catechin described earlier. Many of these metabolites are rare flavonoid derivatives. Thus, microorganisms are excellent models that simulate metabolic patterns observed in both plants and mammals.

Summary

This review has covered numerous phase I (oxidative and some reductive) and phase II (conjugative) biotransformations of flavonoids catalyzed by microbial and some plant enzymes. The rich array of reactions observed with these substrates include hydroxylation, dehydrogenation, methylations, glycosylations, sulfation, and A-, B-, and C-ring degradations. Much information has been gathered into this review, and Table S1 (Supporting Information) was assembled to link interested readers to substrates, useful biocatalysts, and the types of reactions observed. Assembling this table required editorial license to simplify, especially where numerous organisms were screened or used. In these cases, only potentially useful reactions obtained in good yield were listed. We hope that this review and the summary table may encourage others to pursue further microbial and enzymatic biotransformations of flavonoids.

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Supporting Information Available: Summary table of microbial and enzymatic reactions on flavonoids. This information is available free of charge via the Internet at http://pubs.acs.org.

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