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# Flavonol synthase from *Citrus unshiu* is a bifunctional dioxygenase

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Dedicated to Meinhart H. Zenk on the occasion of his 70th birthday

**Abstract**

Flavonol synthase was classified as a 2-oxoglutarate-dependent dioxygenase converting natural (2*R*,3*R*)-dihydroflavonols, i.e. dihydrokaempferol, to the corresponding flavonols (kaempferol). Flavonol synthase from *Citrus unshiu* (Satsuma mandarin), expressed in *Escherichia coli* and purified to homogeneity, was shown to accept also (2*S*)-naringenin as a substrate, producing kaempferol in high yield and assigning sequential flavanone 3β-hydroxylase and flavonol synthase activities to the enzyme. In contrast, dihydrokaempferol was identified as the predominant product from assays performed with the unnatural (2*R*)-naringenin as substrate. The product which was not converted any further on repeated incubations was identified by $^1$H NMR and CD spectroscopies as (−)-*trans*-dihydrokaempferol. The data demonstrate that *Citrus* flavonol synthase encompasses an additional non-specific activity *trans*-hydroxylating the flavanones (2*S*)-naringenin as well as the unnatural (2*R*)-naringenin at C-3.


*Keywords:* *Citrus unshiu*; Rutaceae; Flavonoid biosynthesis; Flavonol synthase; Dihydroflavonol hydroxylase; Bifunctional dioxygenase

## 1. Introduction

Numerous flavonoids accumulate constitutively or upon inductive challenge in spermatophytic plants (Koes et al., 1994; Deboo et al., 1995; Harborne and Williams, 2000), and remarkable bioactivities have been ascribed to some of these metabolites including those in *Citrus* (Benavente-Garcia et al., 1997; Haenen et al., 1997). All flavonoids derive from the flavanone (2*S*)-naringenin which is formed from 4-coumaroyl-CoA and three units of malonyl-CoA by the consecutive activities of chalcone synthase and chalcone isomerase (Heller and Forkmann, 1994). Three branch-point enzymes convert (2*S*)-naringenin to flavones, dihydroflavonols and anthocyanidins, respectively (Fig. 1): flavone synthase (FNS) catalyzes the desaturation to apigenin (Lukačin et al., 2001; Martens and Forkman, 1999; Martens et al., 2001), while flavanone 3β-hydroxylase (FHT) leads to dihydrokaempferol (Lukačin and Britsch, 1997; Lukačin et al., 2000a) which can be reduced subsequently by dihydroflavonol 4-reductase (DFR) to a leucoanthocyanidin on the route to catechins and anthocyanidins (Fig. 1; Heller and Forkmann, 1994). Further two enzymes, flavonol synthase (FLS) and anthocyanidin synthase (ANS), may oxidize dihydroflavonols to flavonols or leucoanthocyanidin to anthocyanidin (Fig. 1). With the notable exception of ANS (Welford et al., 2001), these enzymes were assumed to possess substrate and product stereospecificities.

FLS was reported initially from irradiated parsley cells as a soluble, $Fe^{II}$/2-oxoglutarate-dependent dioxygenase, requiring ascorbate for full activity (Britsch et al., 1981), and detected subsequently in extracts from *Matthiola incana* (Spribille and Forkmann, 1984), *Petunia hybrida* (Forkmann et al., 1986), *Dianthus caryophyllus*

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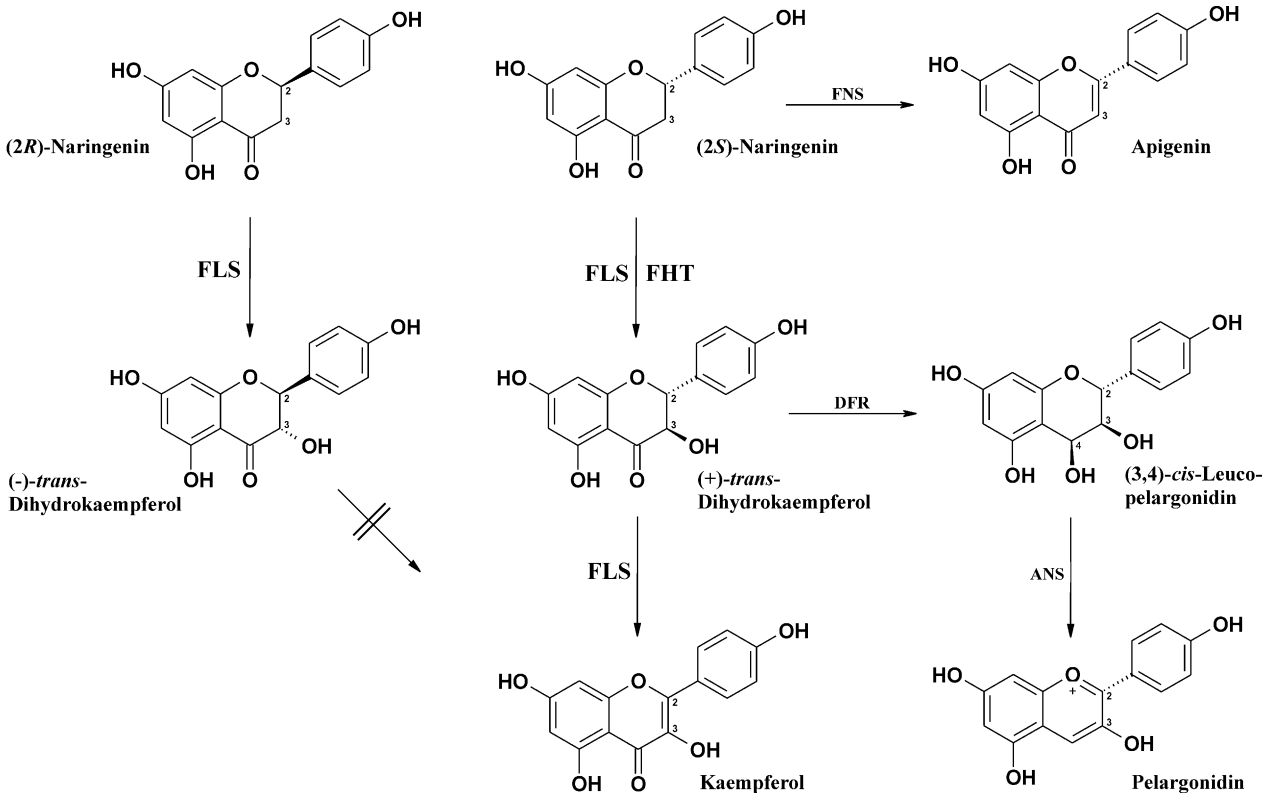


Fig. 1. Reactions in the flavonoid pathway catalyzed by flavanone 3β-hydroxylase (FHT), flavone synthase (FNS), flavonol synthase (FLS), dihydroflavonol-4-reductase (DFR) and anthocyanidin synthase (ANS). FHT catalyzes the 3β-hydroxylation of (2*S*)-naringenin only. FLS converted (2*S*)- or (2*R*)-naringenin to the corresponding *trans*-dihydrokaempferol in addition to the oxidation of (+)-*trans*-dihydrokaempferol, but not that of (−)-*trans*-dihydrokaempferol, to kaempferol.

(Forkmann, 1991) and *Citrus unshiu* (Moriguchi et al., 2002). Meanwhile, the FLS cDNA from *Petunia hybrida* was cloned and used for antisense transformation of petunia and tobacco which intensified the level of flower pigmentation (Holton et al., 1993). More recently, the FLS from *Citrus unshiu* was cloned (Moriguchi et al., 2002), expressed in *E. coli*, and the first kinetic and structural parameters of the recombinant enzyme were elaborated (Wellmann et al., 2002). These studies suggested that the FLS is capable of catalyzing more than one reaction, and, therefore, the activities of *Citrus* FLS with (2*S*)- and (2*R*)-naringenin were examined.

## 2. Results

### 2.1. FLS activity with dihydroflavonol subtrates

FLSs were considered to specifically oxidize 3β-dihydroflavonols to the corresponding flavonols (Holton et al., 1993; Wisman et al., 1998). Therefore, authentic (+)-(2*R*,3*R*)-dihydrokaempferol (Fig. 1), which had been prepared enzymatically from (2*R*/*S*-naringenin) (Britsch and Grisebach, 1986), was incubated with the pure, recombinant FLS from *Citrus unshiu* (Wellmann et al., 2002) under standard assay conditions, and the product was firmly established as kaempferol by comparative cellulose TLC in solvent systems 1 (Fig. 2, lanes 1–5) and 2 (data not shown) with a reference sample and staining with Naturstoffreagenz A (Markham, 1989). The recombinant enzyme also accepted commercial (+)-(2*R*,3*R*) dihydroquercetin as a substrate, and the reaction product was identified as quercetin by its mobilities on RP-HPLC and cellulose TLC in comparison to an authentic standard as well as by EI-MS and MALDI-TOF-MS analyses (Wellmann et al., 2002) (data not shown).

### 2.2. Activity assays with (2S)-naringenin

FLS belongs to the class of 2-oxoglutarate-dependent dioxygenases and shares partial sequence similarity with related enzymes, i. e. FHT (or ANS). In order to distinguish these activities, we also employed pure (2*S*)-naringenin in the FLS assays. Unexpectedly, this compound was accepted and converted almost completely to kaempferol under the conditions of the assay (Fig. 2, lane 6). Trace amounts of dihydrokaempferol were also isolated from these assays suggesting that (2*R*,3*R*)-dihydrokaempferol is an intermediate on the route from

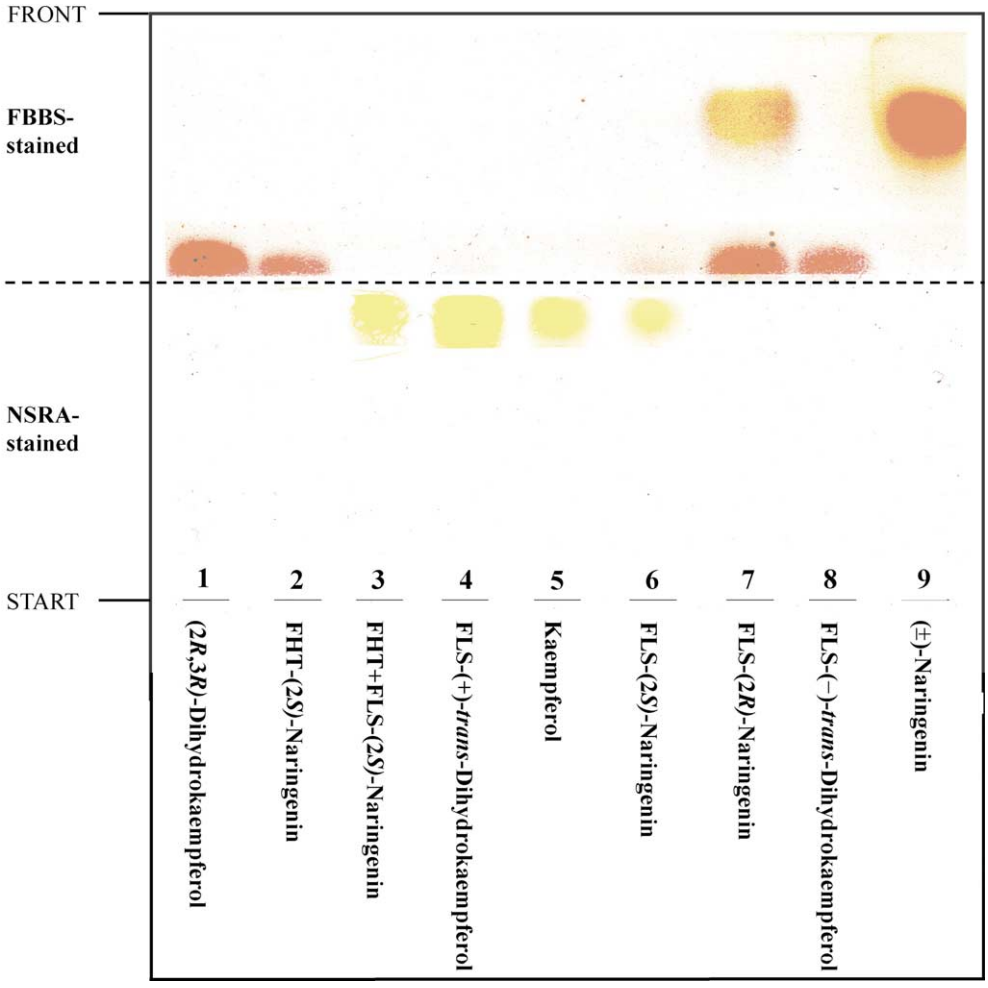


Fig. 2. Chromatography of products isolated from FLS or FHT assays. The incubations (150 μl total) were carried out at 37 °C for 30 min in potassium phosphate buffer pH 6.0, and the flavonoids were extracted with ethylacetate, subjected to cellulose thin-layer chromatography in trichloromethane/acetic acid/water 10:9:1 (v/v/v) and spotted under ultraviolet irradiation (254 nm). The flavonoids were subseqently stained by spraying the plates partly with 1% Naturstoffreagenz A (NSRA, bottom) and 0.1% aqueous fast-blue-B salt (FBBS, top) followed by exposure to ammonia vapors. Authentic (2*R*,3*R*)-dihydrokaempferol (lane 1) and commercial kaempferol (lane 5) as well as (±)-naringenin (lane 9) served as references. The FHT incubation of (2*S*)-naringenin (lane 2) generated *trans*-dihydrokaempferol (total conversion of 10 nmol in 30 min), while the combined incubation with FHT and FLS (lane 3) produced kaempferol under these conditions. Incubation of the pure recombinant FLS with either 10 nmol authentic (+)-*trans*-dihydrokaempferol (lane 4) or 10 nmol (2*S*)-naringenin (lane 6) also yielded kaempferol. However, the incubation of FLS with (2*R*)-naringenin (lane 7) resulted in the formation of dihydrokaempferol only, which was not converted any further on prolonged incubation (up to 1 h) with additional FLS (lane 8).

(2*S*)-naringenin to kaempferol. The assumption was supported by time course studies which provisionally assigned bifunctional FHT/FLS activities to the *Citrus* FLS.

### 2.3. Assays employing (2*R*)-naringenin

Control incubations were carried out in parallel with (2*R*)-naringenin (Fig. 1) isolated from commercial (±)-naringenin by chiral HPLC on a cellulose triacetate-based stationary phase (Krause and Galensa, 1991). The recombinant FLS converted this unatural substrate to a product that could not be distinguished chromatographically from reference (2*R*,3*R*)-dihydrokaempferol (Fig. 2, lane 7). However, only traces of kaempferol were recovered from these assays, ruling out that the product was a true FLS substrate. The lack of conversion was confirmed by extended incubations (up to 1 h) of the isolated product with repeated addition of FLS (Fig. 2, lane 8). Therefore, preparative incubations were conducted to collect about 1 mg of the product which was thoroughly purified by cellulose TLC in solvent system 2, eluted with methanol and subjected to $^1$H NMR spectroscopy. The pattern of resonances fully matched the pattern of authentic (2*R*,3*R*)-dihydrokaempferol (Heller et al., 1985; Britsch and Grisebach, 1986). In particular, a doublet at

5.05 ppm ($J = 11.37$ Hz) was assigned to H-2, and H-3 caused a quartet at 4.6 ppm ($J = 11.37$ and 6.23 Hz) due to additional coupling with the 3-hydroxyl proton, thus verifying the 2,3-*trans*-configuration (Fig. 1). Plants form exclusively (+)-*trans*-dihydrokaempferol, corresponding to the (2*R*,3*R*)-configuration, from (2*S*)-naringenin, but $^1$H-NMR-spectroscopy does not distinguish (+)-*trans*- from (−)-*trans*-dihydrokaempferol. The negligible rate of conversion of the product from (2*R*)-naringenin suggested that aberant (−)-*trans*-dihydrokaempferol had been formed in the FLS incubations. This assumption was finally confirmed by CD spectroscopy which revealed an ellipticity of the product opposite to that of authentic (2*R*,3*R*)-dihydrokaempferol (Fig. 3).

## 3. Discussion

The enzymes of the highly branched flavonoid pathway are generally considered to show narrow substrate specificities, which is presumably essential for the metabolic grid. Nevertheless, ANS (Fig. 1) from *Arabidopsis* has recently been shown not to follow this rule by catalyzing in vitro the desaturation of *trans*-dihydroquercetin to quercetin (Turnbull et al., 2000). Furthermore, the ANS formed *cis*-dihydrokaempferol as a major product in assays conducted with (±)-naringenin (Welford et al., 2001), which clearly identified ANS as a non-specific dioxygenase. FLSs from various plant sources share 50–60% sequence similarity at the polypeptide level with ANSs (accessed to EMBL Library or National Library of Medicine and National Institute of Health, Bethesda, MD, USA), while the similarity to other 2-oxoglutarate-dependent dioxygenases of the flavonoid pathway, i.e. FHT or FNS I, are much lower (less than 35%). These differences might contribute to the degree of catalytic stringency observed with different substrates.

The lack of specificity of FLS is puzzling, because only 3β-dihydroflavonols had been reported as substrates. The fact that (2*S*)- or (2*R*)-naringenin is accepted as a substrate might predict that in both instances the 3β-hydroxylation yields an intermediate on the route to flavonols. In case of (2*R*)-naringenin this would imply the formation of *cis*-dihydrokaempferol which had been reported to isomerize readily to the *trans*-configuration (Welford et al., 2001) upon work-up (TLC purification). While the $^1$H NMR spectroscopy merely assigns the 2,3-*trans*-configuration, the assumption is incompatible with the results from CD spectroscopy (Fig. 3). Overall, the *Citrus* FLS is capable of *trans*-hydroxylating (2*S*)- or (2*R*)-naringenin at C-3 in addition to the desaturation of (2*R*,3*R*)-dihydroflavonols to flavonols, and the low substrate specificity may reflect an incomplete evolution of the FLS as was proposed for ANS (Turnbull et al., 2000). This does not exclude the existence of FLS isoforms with different substrate specificities in *Citrus unshiu* (Moriguchi et al., 2002). For comparison, we re-examined the *Petunia* FHT (Lukačin and Britsch, 1997; Lukačin et al., 2000a,b,c) and corroborated its narrow specificity for (2*S*)-naringenin. Thus, we propose two categories of flavonoid 2-oxoglutarate-dependent dioxygenases, enzymes of narrow (FHT) and broad substrate specificity (ANS and FLS). The specificity of FNS I is under investigation.

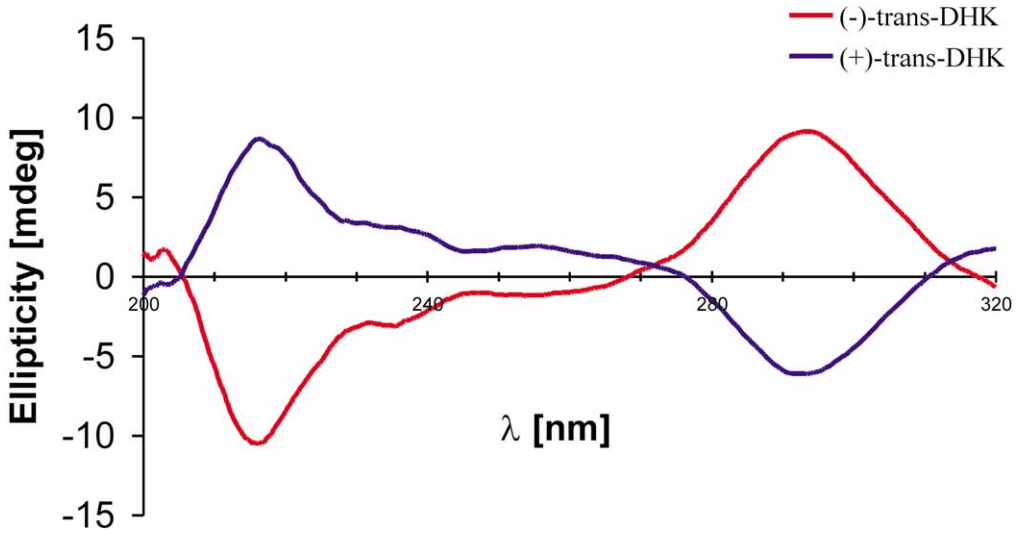


Fig. 3. Mirror-image circular dichroism spectra recorded for authentic (+)-*trans*-dihydrokaempferol and the (−)-*trans*-dihydrokaempferol isolated from FLS incubations with (2*R*)-naringenin. The compounds were dissolved in methanol, and the concentrations were adjusted to about 0.2 μg/μl. The ellipticities are expressed in millidegrees.

## 4. Experimental

### 4.1. Substrates and products

The flavonoid substrates (2*R*/*S*)-naringenin, (+)-(2*R*,3*R*)-dihydroquercetin, quercetin and kaempferol were purchased from Roth (Karlsruhe, Germany). Authentic (+)-(2*R*,3*R*)-dihydrokaempferol was collected from preparative FHT incubations with (2*R*/*S*)-naringenin (Britsch and Grisebach, 1986). The identity of the product was verified by chromatographic and spectroscopic comparison (ultraviolet absorbance, $^1$H NMR and ORD) with the reference compound isolated from flowers of rose-coloured and white mutants of *Alcea rosea* L (Aromadendrin), whose stereoconfiguration had been established by spectroscopy (Britsch and Grisebach, 1986). Stereochemically pure (2*S*)-naringenin had been produced by chalcone synthase/chalcone isomerase incubations and confirmation of the optical purity by ORD spectroscopy (Britsch and Grisebach, 1986). (2*R*)-Naringenin was chromatographically isolated from commercial (2*R*/*S*)-naringenin as described elsewhere (Krause and Galensa, 1991). Briefly, 180 mg of (2*R*/*S*)-naringenin were dissolved in 2 ml methanol and applied to a 26 × 295 mm column, packed with microcrystalline cellulose triacetate, 15–25 μm (Merck, Darmstadt, Germany), and preequilibrated with methanol. The pure (2*S*)- and (2*R*)-enantiomers of naringenin were isolated successively by isocratic elution at 8 ml/min. The separation was monitored by ultraviolet absorbance (254 nm) which clearly distinguished the peak and side fractions. The peak fractions were collected, the solvent was removed in vacuo at room temperature, and (2*R*)-naringenin was obtained as colourless crystalline powder.

### 4.2. Analytical methods

FHT or FLS activities were measured as described previously (Lukačin and Britsch, 1997; Lukačin et al., 2000a,b,c; Wellmann et al., 2002). The enzyme products were analyzed by chromatography on cellulose thin-layer plates (Merck, Darmstadt, Germany) with the solvents (1) trichloromethane/acetic acid/water (10:9:1, v/v/v) or (2) 15% aqueous acetic acid. Naringenin and dihydrokaempferol were spotted under ultraviolet irradiation (254 nm) and by spraying with 0.1% aqueous fast-blue-B salt and exposure to ammonia vapors. The flavonols kaempferol and quercetin were visualized on air-dried plates with 1% diphenyl-boric acid-ethanolamine-complex in methanol (Naturstoffreagenz A). $^1$H NMR spectra were recorded on a 500 MHz spectrometer (Bruker) with tetramethylsilane as an internal standard (Heller et al., 1985; Britsch and Grisebach, 1986). CD spectroscopy was done on a Jasco-720 spectropolarimeter (Tokyo, Japan) interfaced to an 486/33 PC and controlled by Jasco software (Lukačin et al., 2000b). The instrument was calibrated with 0.06% ammonium d-10-camphor sulfonate and the dihydrokaempferol spectra were recorded in methanol as described elsewhere (Gaffield, 1970).

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