# Distinct Substrate Specificity of Dihydroflavonol 4-Reductase from Flowers of *Petunia hybrida*

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Dihydroflavonol 4-reductase from *Petunia* flowers catalyzes the reduction of dihydroquercetin to leucocyanidin and, in particular, of dihydromyricetin to leucodelphinidin, whereas reduction of the simple dihydroflavonol dihydrokaempferol to leucopelargonidin could not be observed. This special substrate specificity of dihydroflavonol 4-reductase is most probably the reason for the observations that delphinidin derivatives are the main end products of anthocyanin biosynthesis in *Petunia* flowers, whereas anthocyanins based on pelargonidin are rarely found and, if present, are only formed in very small amounts.

## Introduction

Dihydroflavonol 4-reductase, which is involved in anthocyanin biosynthesis, catalyzes the stereospecific conversion of (+)-dihydroflavonols to flavan-3,4-cis-diols (leucoanthocyanindins). The latter compounds are the immediate precursors for the respective anthocyanidins (Fig. 1) [1, 2].

The enzymes from flowers of *Matthiola* [2], *Callistephus* [3], *Sinningia* [4], *Dianthus* (Forkmann, unpublished) and *Dahlia* (Grisebach, personal communication) were found to use dihydrokaempferol, dihydroquercetin and dihydromyricetin as substrate for the reduction reaction, to the respective leucoanthocyanidins. Dihydromyricetin is even reduced by enzyme extracts from flowers which naturally lack delphinidin derivatives. The substrate specificity of dihydroflavonol 4-reductase seemed therefore to be not essentially influenced by the B-ring hydroxylation pattern of dihydroflavonols. Enzymatic studies with *Petunia* flowers extracts revealed now, however, that the dihydroflavonol 4-reductase of this plant exhibits a high substrate specificity with regard to the B-ring substitution pattern.

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## Materials and Methods

### *Plant material*

The studies included lines, which lack flavonoid 3′- and flavonoid 3′,5′-hydroxylase (recessive htl and hfl), and the commercial strains "Red Titan" and "Blue Titan" (Benary, Hann. Münden, FRG) which possess 3′-hydroxylase activity (dominant Htl) and 3′,5′-hydroxylase activity (dominant Hfl), respectively [5, 6]. The lines were cultivated in a greenhouse.

### *Chemicals and labelled substrates*

(+)-Dihydroflavonols and 4-coumaroyl-CoA were from our laboratory collection. Leucoanthocyanindins were kind gifts from W. Heller (Neuherberg, FRG) and L. Britsch (Freiburg, FRG). Labelled (+)-dihydroflavonols (3.09 GBq/mmol) were prepared enzymatically from [$^{14}$C]malonyl-CoA (Amersham-Buchler, Braunschweig, FRG) and 4-coumaroyl-CoA as described earlier [2].

### *Enzyme preparation and enzyme assay*

The preparation of crude extracts from buds and young flowers and the gel filtration of the extracts was performed as described [2] with the exception that glycerol was omitted. The standard enzyme assay contained in a total volume of 100 µl: 0.03 nmol radioactive substrate (87 Bq), 500 nmol NADPH in 20 µl water and 112 µg protein in 0.1 M McIlvaine buffer, pH 6.8, with 2.8 mmol 2-mercaptoethanol. Incubation was carried out for 30 min at 25 °C. The reaction mixture was immediately extracted twice with ethyl acetate (80 µl, 80 µl) and the extract chromatographed on cellulose plates (Schleicher & Schüll, Dassel, FRG) with the solvent system chloroform/acetic acid/water (10:9:1). Because at least leucodelphinidin is poorly extracted from the reaction mixture, 50 µl of the enzyme assay were also directly spotted on a cellulose plate and separated as described above. After chromatography the plates were scanned for radioactivity. Radioactive zones were stripped of [7] and counted in Aqualuma.

Analytical methods were performed as described earlier [2, 3].

## Results and Discussion

Because the synthesis of labelled dihydroflavonols with different B-ring hydroxylation pattern is expen-

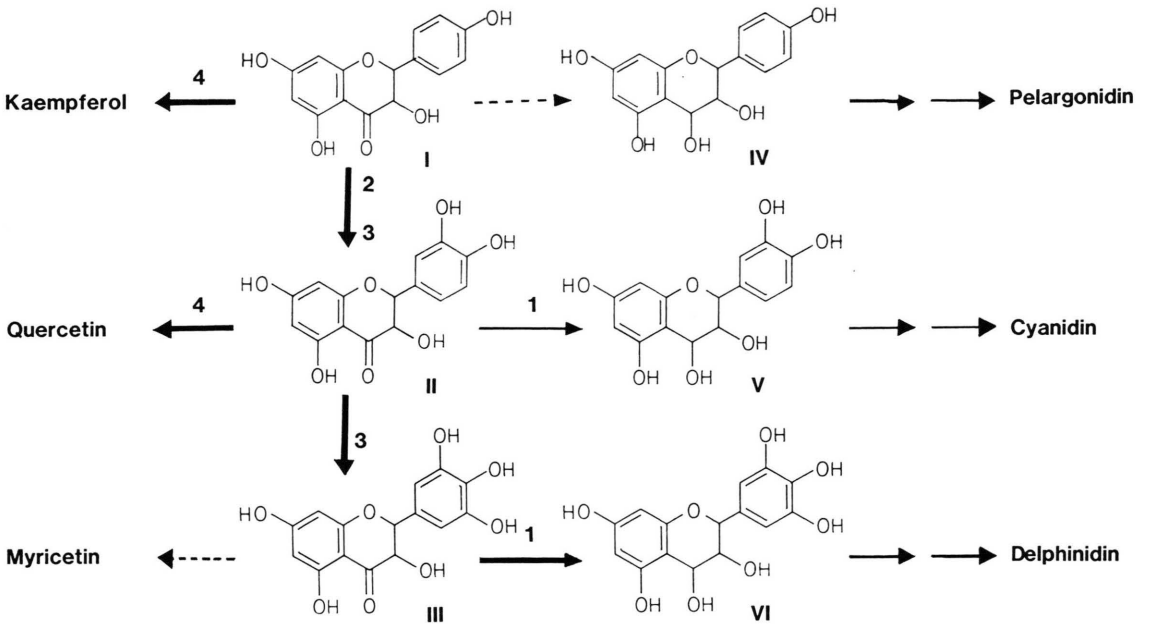


Fig. 1. Enzymatic formation of leucoanthocyanidins and anthocyanidins, respectively, and of flavonols from dihydroflavonols with different B-ring hydroxylation pattern in *Petunia* flowers.
**I** = dihydrokaempferol; **II** = dihydroquercetin; **III** = dihydromyricetin; **IV** = leucopelargonidin; **V** = leucocyanidin; **VI** = leucodelphinidin.
**1** = dihydroflavonol 4-reductase; **2** = flavonoid 3′-hydroxylase; **3** = flavonoid 3′,5′-hydroxylase; **4** = flavonol synthase.
——▶ Main pathway;
-----▶ minor pathway.

sive and time consuming, dihydrokaempferol as the simplest compound is generally used for the characterisation of the reductase reaction. This substrate is also converted by flavonoid 3′- and flavonoid 3′,5′-hydroxylase with NADPH as co-factor (Fig. 1). In *Petunia,* activity of these two hydroxylases can be excluded by the use of a line with recessive alleles of the genes Htl and Hfl [5, 6].

When flower extracts of such a line were incubated with [$^{14}$C]dihydrokaempferol in the presence of NADPH, no formation of the respective flavan-3,4-diol leucopelargonidin was observed. Dihydroflavonol 4-reductase activity could also not be demonstrated by variation of the enzyme preparation and of the assay conditions including different pH-values, temperature and time of incubation and NADPH concentration.

Commercial strains often exhibit much higher activities for enzymes involved in flavonoid biosynthesis than genetically defined mutant lines. For further studies we therefore prepared enzyme extracts from the strains "Red Titan" and "Blue Titan", which we normally use for the synthesis of labelled flavonoids. In fact, we could now demonstrate dihydroflavonol 4-reductase activity. With enzyme preparations from flowers of "Red Titan" containing flavonoid 3′-hydroxylase activity, conversion of dihydrokaempferol to dihydroquercetin and to some leucocyanidin was observed. And with flower extracts from "Blue Titan", which possess flavonoid 3′,5′-hydroxylase activity, some dihydroquercetin, dihydromyricetin and, in particular, leucodelphinidin were found to be formed from dihydrokaempferol. But reduction of dihydrokaempferol to the respective flavan-3,4-diol leucopelargonidin was again not observed (Table I). Thus, dihydroquercetin and dihydromyricetin but not dihydrokaempferol seems to be a suitable substrate for the reductase reaction.

In order to confirm this assumption, we prepared labelled dihydroquercetin and dihydromyricetin and incubated each with NADPH and flower extract of "Red Titan". As expected, dihydroquercetin was reduced to leucocyanidin and dihydromyricetin to leucodelphinidin. Again the reduction rate of di-

Table I. Amount of products formed from dihydroflavonols by action of flavonoid 3′-hydroxylase or 3′,5′-hydroxylase and dihydroflavonol 4-reductase.

| Line | Substrate | Conversion rate [%] of the substrate to | | | | |
|---|---|---|---|---|---|---|
| | | DHQ | DHM | LPg | LCy | LDp |
| "Red Titan" | DHK | 42.4 | – | – | 5.8 | – |
| "Blue Titan" | DHK | 12.6 | 16.7 | – | – | 41.5 |
| "Red Titan" | DHQ | – | – | – | 13.5 | – |
| "Red Titan" | DHM | – | – | – | – | 80.4 |

DHK = dihydrokaempferol; DHQ = dihydroquercetin; DHM = dihydromyricetin; LPg = leucopelargonidin; LCy = leucocyanidin; LDp = leucodelphinidin.

hydromyricetin was considerably higher than that of dihydroquercetin (Table I). Similar differences for the both substrates were found with enzyme extracts from flowers of "Blue Titan". Moreover, with dihydroquercetin and, in particular, dihydromyricetin as substrate, dihydroflavonol 4-reductase activity could now also be demonstrated in flower extracts from lines which lack flavonoid 3′- and flavonoid 3′,5′-hydroxylase activity.

These results prove that, in contrast to the flowering plants mentioned above, the dihydroflavonol 4-reductase from *Petunia* flowers exhibits a distinct substrate specificity. Dihydromyricetin (3′,4′,5′-OH) is highly reduced to leucodelphinidin, whereas dihydroquercetin (3′,4′-OH) is only poorly converted to leucocyanidin, and dihydrokaempferol (4′-OH) is not used as substrate for the reduction reaction (Fig. 1).

This special substrate specificity is in complete agreement with the observation that in the flowers of *Petunia hybrida* derivatives of kaempferol and quercetin and, with regard to anthocyanins, delphinidin derivatives are the main endproducts of flavonoid biosynthesis [8, 9]. Higher amounts of cyanidin derivatives were only formed in the absence of flavonol synthase, which competes with dihydroflavonol 4-reductase for dihydroquercetin as common substrate (8). In the presence of both enzyme activities, dihydroquercetin is mainly converted to quercetin (Fig. 1). Such a competition does not occur for dihydrokaempferol. In the presence of both flavonol synthase and dihydroflavonol 4-reductase activity, dihydrokaempferol is highly converted to kaempferol but not reduced to leucopelargonidin. In flowers, which lack flavonol synthase or exhibit a reduced activity, dihydrokaempferol is accumulated (Fig. 1) [9].

From these results, it can be concluded, that the substrate specificity of dihydroflavonol 4-reductase is apparently the only reason for the rare occurrence and, if present, for the small amounts of pelargonidin derivatives in the flowers of this plant [10]. This conclusion is further confirmed by feeding experiments on flowers of *Petunia*, where leucopelargonidin was found to be a suitable and strong precursor for the formation of pelargonidin glucosides (Forkmann, unpublished).

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