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CONVERSION OF HYDROXYLATED AND METHYLATED DIHYDROFLAVONOLS INTO ANTHOCYANINS IN A WHITE FLOWERING MUTANT OF *PETUNIA HYBRIDA*

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Abstract—A 3′, 4′-dihydroxy or a 3′, 4′, 5′-trihydroxy substitution pattern of dihydroflavonols is required for their conversion into the corresponding anthocyanins in a white flower of *Petunia hybrida*. The presence of a 5-hydroxyl group is not required. B-ring methylated dihydroflavonols were not converted into the corresponding anthocyanins. In case of a 4′-methoxy substituted dihydroflavonol a 4′-hydroxyanthocyanin is obtained, suggesting demethylation of this compound. The conversion of synthetic (±)-*trans*-2,3-dihydroflavonols into anthocyanins proceeded almost as well as with natural compounds. The results demonstrate that the cinnamic acid starter hypothesis for the origin of B-ring substituents is not correct for B-ring methylation.

INTRODUCTION

Investigations of flavonoid biosynthesis with mutant strains of higher plants has revealed additional knowledge that could not be obtained from radiotracer experiments [1, 2]. Wong and Francis [3–5] examined the flavonoids which accumulated under various conditions in several mutants of *Trifolium subterraneum* and demonstrated that there was genetic control of the biosynthetic reactions involved in the overall flavonoid pathway.

A particularly useful application of mutants in such studies is the complementation technique [6, 7]. Some white flowering mutants of higher plants, which are blocked in the biosynthetic pathway leading to anthocyanins, are capable of synthesizing these pigments when appropriate precursors are supplied to the flowers. For example, Stickland and Harrison [8, 9] observed that white flowering genotypes of *Antirrhinum majus*, *Streptocarpus hybridus*, *Anemone coronaria* and *Primula obconica* produced anthocyanins when the flower stems were placed in aqueous solutions of the expected flavanone or dihydroflavonol precursors. Isolated corollas of a white flowering mutant of *Petunia hybrida* also produced anthocyanins when incubated with dihydroflavonols, but they did not do so when incubated with flavanones or flavonols [7]. From such experiments a more detailed knowledge about the blocked steps in the anthocyanin pathway has been obtained in various genotypes of *Antirrhinum* and *Petunia*.

Immature corollas of a white *Petunia* mutant, which is a homozygous recessive for the anthocyanin gene An 3, synthesize a mixture of cyanidin and delphinidin glycosides when incubated with a solution of (+) dihydroquercetin (3, 3′, 4′ 5, 7-pentahydroxyflavanone, cf. **1a**, Scheme 1 [10]. The synthesis of delphinidin derivatives, indicates the potential to introduce a third hydroxyl group into the B-ring of dihydroquercetin. Probably dihydromyricetin (Scheme 1, **2**) is formed as an intermediate.

It has been suggested that in *Petunia hybrida*, the substitution pattern of the B-ring of anthocyanins is determined at the cinnamic acid stage [1, 2]. If this is true, substituted dihydroflavonols may be expected to act as precursors only of those anthocyanins with an analogous substituted B-ring when fed to the appropriate white flowering mutants.

In the present work we have examined this hypothesis by feeding differently substituted natural and synthetic dihydroflavonols to corollas of the white flowering mutant W 39 of *Petunia hybrida*. This mutant is a homozygous recessive for the anthocyanin gene An 3 and is blocked before dihydroflavonol formation. It has the same properties as the mutant W 18 used in our earlier experiments [7, 10].

RESULTS

Incubation of isolated corollas of mutant W 39 in the presence of dihydroflavonols resulted in several cases in reddish or bluish pigmentations (Table 1). Synthetic dihydroquercetin-7-*O*-glucoside (Scheme 1, **1b**) proved to be a slightly less active stimulant of pigment formation than the natural compound. In both cases cyanidin and some delphinidin were detected after hydrolysis. Application of synthetic dihydromyricetin (Scheme 1, **2**) caused a more bluish pigmentation than did dihydroquercetin, and the $\lambda_{\max}^{\mathrm{MeOH/HCl}}$ of the pigment was shifted to a slightly longer wavelength in this case. Acid hydrolysis of the pigment released delphinidin and traces of petunidin and malvidin.

We also tested two naturally occurring 5-deoxydihydroflavonols, (−) dihydrofisetin and (+) dihydrorobinetin. Only the (+) latter (Scheme 1, **3**) was able to induce pigmentation in the corollas and in this case the anthocyanidin obtained after acid hydrolysis was

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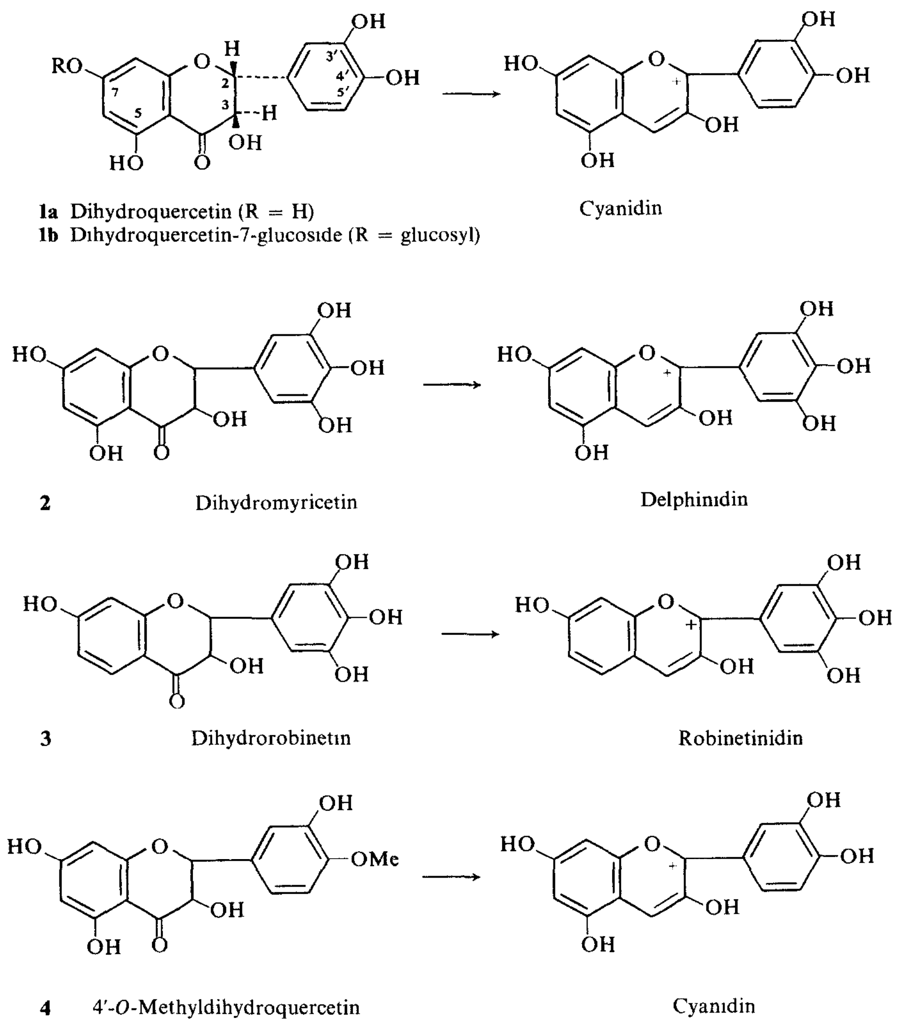

1a Dihydroquercetin (R = H)
1b Dihydroquercetin-7-glucoside (R = glucosyl)
Cyanidin

2 Dihydromyricetin
Delphinidin

3 Dihydrorobinetin
Robinetinidin

4 4′-*O*-Methyldihydroquercetin
Cyanidin

Scheme 1. Conversion of dihydroflavonols into anthocyanidins in corollas of the white flowering mutant W 39 of *Petunia hybrida*.

identified as robinetinidin. Only 4′-*O*-methyldihydroquercetin (Scheme 1, **4**) of the B-ring methylated dihydroflavonols gave rise to a weak pigmentation in the corollas. The anthocyanidin obtained from this experiment was not the expected 4′-*O*-methylcyanidin but cyanidin itself, showing that *O*-demethylation must have occurred.

As can be seen in Table 1, the largest amounts of pigments were formed with dihydroquercetin and dihydromyricetin. The application of flavonone glycosides (naringenin-7-glucoside or eriodyctiol-7-glucoside) or flavonols (quercetin) did not enhance the anthocyanin level above the control.

DISCUSSION

It has been shown that *trans* 2,3-dihydroflavonols e.g. (+) dihydroquercetin) and not the *cis* isomers are intermediates in anthocyanin biosynthesis [11, 12]. This means that if a synthetic (±) dihydroflavonol is administered to an appropriate white flower conversion into anthocyanins may not occur for both enantiomers to the same extent. It is likely that only the one whose absolute configuration corresponds to that of (+) dihydroquercetin will be converted. Indeed the presence of 50 % of the inactive enantiomer in the case of synthetic (±) compounds may cause some inhibition. To test this hypothesis, naturally occurring and synthetic dihydroquercetin-7-glucose were compared in our test system at equal concentrations. Although the synthetic compound was about $\frac{1}{3}$rd less readily converted, the inactive enantiomer apparently did not inhibit the reaction. Further evidence for the view that only the (+) enantiomers of dihydroflavonols can be converted into anthocyanins may however, be derived from the observed inactivity of (−) dihydrofisetin.

The readily conversion of dihydromyricetin into delphinidin and its *O*-methyl derivatives petunidin and malvidin suggest that this dihydroflavonol is a ready precursor of delphinidin. This implies that B-ring hydroxylations occur prior to the conversion of dihydroflavonols into anthocyanins.

In contrast, B-ring methylated dihydroflavonols were not converted into methylated anthocyanins. The production of some cyanidin from 4′-*O*-methyldihydroquercetin could have been the result of demethylation

Table 1. Anthocyanin synthesis in isolated corollas of the white flowering mutant W 39 of *Petunia hybrida* after incubation with solutions of dihydroflavonols in aqueous culture medium

Dihydroflavonol (0.5 mM)	λ_{max} (nm)	Anthocyanidin produced	$A(\lambda_{max})$	Visible pigmentation
(+)-Dihydroquercetin-7-glucoside (natural)	530	Cyanidin	0.37 ± 0.04	+ + +
		Delphinidin		
(±)-Dihydroquercetin-7-glucoside (synthetic)	530	Cyanidin	0.24 ± 0.04	+ +
		Delphinidin		
(−)-Dihydrofisetin (natural)	—	—	0.06 ± 0.04	–
(+)-Dihydrorobinetin (natural)	524	Robinetinidin	0.21 ± 0.04	+ +
Dihydromyricetin (synthetic)	537	Delphinidin	0.34 ± 0.06	+ + +
		Petunidin		+
		Malvidin		±
(+)-Dihydroquercetin (natural)	530	Cyanidin	0.87 ± 0.09	+ + +
		Delphinidin		+ +
3′-*O*-Methyldihydroquercetin (synthetic)	—	—	0.06 ± 0.04	—
4′-*O*-Methyldihydroquercetin (synthetic)	530	Cyanidin	0.20 ± 0.02	+
Control	—	—	0.07 ± 0.02	—

Anthocyanin content was determined by measurement absorptivity of MeOH–HCl extracts of the corollas at the λ_{max} in the region between 520–540 nm and expressed as $A\ \lambda_{max}$/corolla. As a control, corollas were incubated in medium without flavonoids. Values represent the mean ± standard deviation of 5–10 corollas. Anthocyanidins were detected after acid hydrolysis by co-chromatography with standards on cellulose TLC plates.

prior to anthocyanin formation. Demethylating activity in flowers of *Petunia hybrida* has been reported earlier by Steiner [15]. This result supports earlier evidence from genetic experiments that B-ring methylation is the ultimate step in anthocyanin biosyntheisis in *Petunia* [16].

The conversion of dihydrorobinetin into robinetinidin [17], which does not occur in nature, is interesting. Obviously the natural enzyme system that converts dihydroflavonols into normal anthocyanins can also convert this 5-deoxysubstrate. However, earlier work indicated that dihydrokaempferol (3, 4′, 5, 7-tetrahydroxyflavanone) is not a precursor of pelargonidin in *Petunia* [7]. Therefore only compounds with the 3′, 4′-dihdroxy or the 3′, 4′, 5′-trihydroxysubstituted B-ring substitution pattern can be converted into the corresponding anthocyanins.

The results we have obtained point to the fact that methylation does not occur before the anthocyanin stage and, therefore, are not in agreement with the cinnamic acid starter hypothesis of Hess [1] with regard to the origin of B-ring *O*-methyl groups of anthocyanins in *Petunia hybrida.*

EXPERIMENTAL

Plant material. A clone of the white flowering mutant W 39 of *Petunia hybrida*, homozygous recessive for the gene An 3, maintained in the collection of the Institute of Genetics, University of Amsterdam was cultivated in the greenhouse.

TLC methods. Chromatograms on cellulose plates with fluorescent indicator, (E. Merck, A.G., Darmstadt, Germany), were developed in the solvent systems BAW (*n*-BuOH–HOAc–H_2O, 4:1:5,) CAW ($CHCl_3$–HOAc–H_2O, 10:9:1), 15% HOAc (aq.), Forestal (HAc–H_2O–HCl, 30:10:3) and propanol-2-2N HCl (1:1).

Chemicals. (+) Dihydroquercetin, (+) dihydrorobinetin, (−) dihydrofisetin, hesperidin and myricetin were obtained from Carl Roth A.G., Karlsruhe, Germany. Standard anthocyanidins were provided by Mrs. P. de Vlaming, Institute of Genetics, University of Amsterdam. Dihydroquercetin-7-glucoside was isolated from mutant W 19 of *Petunia hybrida* [7].

Synthesis of flavonoids. Naringenin-7-glucoside was synthesized according to ref. [18]. Dihydroquercetin-7-glucoside. Eriodyctiol-7-glucoside (0.5) g mp 164–167°, synthesized according to ref. [19], dissolved in 10 ml 2N NaOH, was oxidized with 0.5 ml 30% H_2O_2 for 48 hr at 0°, neutralized to pH 4 with 6N HCl and dried under red. press. The residue was treated with 20 ml MeOH and filtered. After removal of the MeOH under red. press. the residue was dissolved in a few ml H_2O. The dihydroflavonol was purified over Solka Floc and polyamide columns by the procedure of ref. [20]. Slow evapn of the aq. soln yielded a yellowish glass that could not be crystallized (mp 175–195°, dec.). R_f BAW: 0.43, R_f CAW: 0.10, λ_{max}^{MeOH} 287 nm, $\lambda_{max}^{MeOH+AlCl_3}$ 311 nm, $\lambda_{max}^{MeOH+NaOAc}$ 311 nm. Acid hydrolysis liberated dihydroquercetin and glucose, identified by TLC [7]. Spectral and R_f data were identical to those for the natural compound. 3′-*O*-Methyldihydroquercetin. Synthetic [20] 3′-methoxy, 7-neohesperidosyloxy, 3, 4′, 5-trihydroxyflavone (30 mg) [20] was hydrolyzed in 0.5 ml 2N HCl in a sealed tube under N_2 for 1 hr at 100°. The aglycone was extracted with peroxide-free Et_2O. The Et_2O fraction was washed with H_2O until neutral. After evapn of the Et_2O, the residue was dissolved in 10 ml H_2O, and freeze-dried. Yield 10 mg, mp 214–218°, R_f BAW: 0.92, CAW: 0.35, λ_{max}^{MeOH} 289 nm. Bisulfite oxidation [21] yielded a flavonol that was identified as isorhamnetin by TLC. 4′-*O*-Methyldihydroquercetin (4′-methoxy, 3, 3′, 5, 7-tetrahydroxyflavanone). Synthetic 4′-methoxy, 7-rutinosyloxy, 3, 3′, 5-trihydroxyflavone (65 mg) [20] was hydrolyzed in 2N HCl by the procedure as mentioned before. The aglycone crystallized from hot H_2O as colourless needles, mp 118–122°, yield 25 mg. λ_{max}^{MeOH} 289 nm, $\lambda_{max}^{MeOH+AlCl_3}$ 316 nm, $\lambda_{max}^{MeOH+NaOAc}$ 327 nm. R_f BAW = 0.92, CAW 0.71, violet colour with Zn/HCl. Bisulfite oxidation [21] yielded a flavonol, R_f 0.03 (15% HOAc), λ_{max}^{MeOH} 368, 255 nm, $\lambda_{max}^{MeOH+NaOMe}$ 413, 320 *sh*, 275 nm, stable spectrum. Dihydromyricetin. Myricetin (390 mg) was reduced by treatment with sodium dithionite [22]. The reaction mixture was worked up according to ref. [23]. The crude product (30 mg) was crystallized from hot H_2O and purified by Sephadex LH-20 column (87 × 4 cm) chromatography in MeOH. From the combined Zn/HCl positive fractions MeOH was removed under red. press. The residue was crystallized twice from hot H_2O and dried for 2 hr at 105°. Yield 25 mg, mp 240–244°, (dec.). λ_{max}^{MeOH} 292 nm, R_f BAW 0.69, CAW 0.16. Found: C 56.6; H 3.5%, calcd for

$C_{15}H_{12}O_8$ C 56.6; H 3.7%. Bisulfite oxidation yielded myricetin, identified by TLC.

Synthetic anthocyanidins. Robinetinidin chloride (R_f Forestal = 0.46, R_f propanol-2–2N HCl (1:1) 0.22) and 4′-*O*-methylcyanidin chloride (R_f Forestal 0.61, R_f propanol-2-2N HCl (1:1) 0.38) were prepared via the ψ-bases from dihydrorobinetin and 4′-methoxy-3, 3′, 5, 7-tetrahydroxyflavanone by the method of ref [24]. 4′-*O*-Methylcyanidin chloride was synthesized on a microscale starting from 1 mg of the flavanone.

Administration of dihydroflavonols to isolated corollas. Corollas obtained from bud stage III of the white flowering mutant W 39 [7] were incubated at 24° in petri dishes (60 × 15 mm) containing 4 ml of a soln of the dihydroflavonol (0.5 mM) in culture medium under aseptic conditions for 72 hr in darkness [7]. The concn of the methoxylated compounds was checked spectrophotometrically using $\varepsilon_{1\,\mathrm{cm}}^{291\,\mathrm{nm}} = 1.8 \times 10^4$

Identification of anthocyanidins. After the incubation period anthocyanins were extracted by shaking each corolla in 3 ml MeOH-HCl 0.1% for 1 hr. The *A* of the solution at the λ_{max} between 520–540 nm was used as a quantitative measure for anthocyanin content [25]. The MeOH–HCl extracts of 5–10 corollas were combined and MeOH was removed under red. press. The residue was dissolved in 1 ml 2N HCl and hydrolyzed under N_2 in a sealed tube for 40 min at 100° The cooled hydrolysate was centrifuged for 8 min at 2000 *g* The released anthocyanidins were extracted from the supernatant by shaking with a few drops of isoAmOH and applied to TLC plates. They were identified by co-chromatography with standard anthocyanidins in the solvent systems forestal and propanol-2–2N HCl (1:1)

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